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Project #1 Multi-System Brain-Motor Performance Assessment

INTRODUCTION

The goal of this research project was twofold:

1) Assemble multimodal human performance laboratory including complex human motor assessment system, 128 channel EEG/ERP, pupilometer/ eyetracking system, and repetitive transcranial magnetic stimulation system.

2) Conduct a pilot research study demonstrating the capabilities of performing multimodal assessment of object retrieval, particularly when those objects may be considered threatening or nonthreatening.

BODY

Task #1: Assemble multimodal human performance laboratory including complex human motor assessment system, 128 channel EEG/ERP, pupilometer/ eyetracking system, and repetitive transcranial magnetic stimulation system.

a. We have purchased each of the pieces of equipment in Task #1, assembled the units, trained to use each of the devices, and have assembled the units for integrative assessments in the Multimodal Human Performance Laboratory (MHPL).

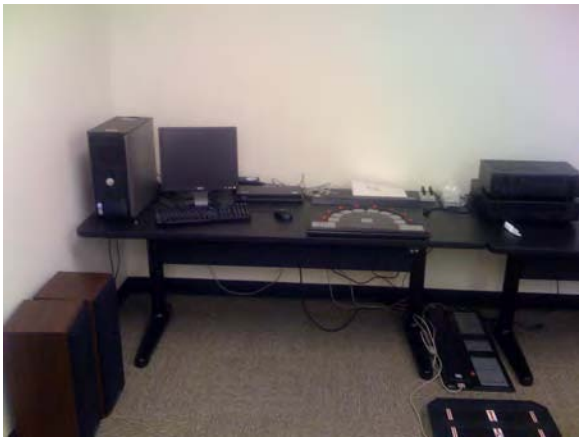
The equipment assembled includes the following

1. repetitive Transcranial Magnetic Stimulation (rTMS) system – The Magstim Super Rapid^2 package which includes a Rapid^2 unit and air cooled coil, dual power supply module, amplifier, coil stand, as well as a double rapid2 air cooled coil and sham cooled double coil. This unit allows for safe delivery of repetitive transcranial magnetic stimulation pulses (including sham administration) in the standard doulble blind, placebo design.

Brainsight TMS "Turnkey" system which includes Brainsight TMS software and the TMS Basic Tracker Kit was also purchased in conjunction with the rTMS system. The Brainsight system allows for targeting of the rTMS pulses to region previously identified with neuroimaging techniques and allows for the optimal, maximal delivery of the rTMS pulses to the targeted brain region.

2. SensoMotoric Instruments (SMI) eyetracker system – One Hi-Speed 1250 eye tracking system was purchased which include a 1250 Hz camera chin rest assembly, 500 Hz binocular extension, operating PC, experiment writing tool, and BeGaze 2 Analysis software package. Also, purchased with this package is the Presentation stimulus presentation package and SpeakAloud attachment for presentation of experimental paradigms that would be compatible with all of the neuroinvestigative platforms in the MHPL.

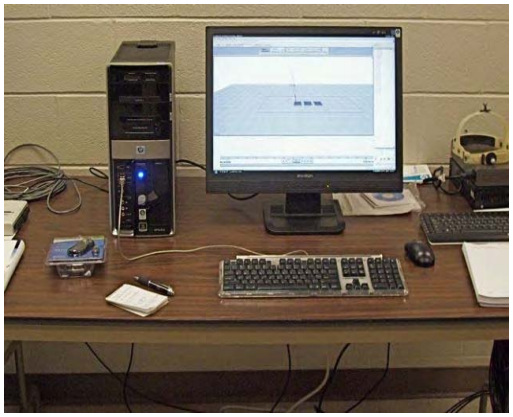
3. Biologics EEG System – The lab contains a Biologics 128 clinical EEG system with differential amplifiers and Orgil stimulus presentation system. The stimuli are presented to the subject on an LCD monitor while a separate computer records onset times and subject responses. The Biologics system is interfaced with a Neuroscan Quik-Cap for data acquisition.



4. Human Motor Performance assessment system – This integrated human performance laboratory includes a central processing and upper extremity motor control unit, lower extremity motor control performance measurement system, postural stability performance capacity measurement system, multiple module server, Gait Mat, electronic height adjustable tables, and Latitude D531 1.6 HZ PC.

5. High-end Immersive Driving/Task Simulation System – This provides a common, controlled environment to provide real-world stressful challenges to cognitive, information processing, and selected neuromotor subsystems and the investigation of a wide variety of human performance issues.

6. Eight camera Human Motion Capture and Analysis System – This system provides for high fidelity capture of full body or selected subsystem human motion in complex tasks. It has been installed in a lab with a 20 x 18 ft data acquisiton region. This will support a broad range of investigations of human performance in medical and non-medical application contexts.



Overall, we have assimilated unique integrated laboratory to assess human cognition and motor performance, with the capability to further integrate the use of the this laboratory with results from neuroimaging (structural MRI, fMRI, diffusion tensor imaging for white matter tract imaging, etc.)

b. Perform a pilot study using the assembled multimodal laboratory with the aims to identify subgroups of normal young controls and veterans with an increased, decreased or normal amplitude P300 response to combat-threatening stimuli and administer 1 Hz. rTMS to each frontal lobe to determine if the threat response is mediated by the right frontal lobe.

The protocol was submitted and received approved by the University of Texas Institutional Review Board in June 2007(see attached University of Texas consent form with IRB approval of the pilot project proposed – {dodrTMSirb.pdf}). The protocol was submitted to the U.S. Army Medical Research and Material Command's Office of Research Protections, Human Research Protections Office, received request for revisions, the revisions were made as directed and the proposal was returned to the to the HRPO. The protocol remains (as of September 2008) at the HRPO still awaiting approval. We were informed that the study could not be performed until we received approval form the HRPO and we have not received approval from that Office.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of the integrated Multimodal Human Performance Laboratory
    - Including
        - repetitive Transcranial Magnetic Stimulation System with Magstim Super Rapid^2 package and BrainSight Software System
        - SensoMotoric Instruments (SMI) Eyetracker system
        - Biologics EEG System
        - Human Motor Performance assessment system

REPORTABLE OUTCOMES

Task #1a. has been accomplished. The Multi-Modal Brain-Motor Performance Laboratory is fully constructed and operational at the University of Texas at Dallas Center for BrainHealth located at 2200 W. Mockingbird Lane, Dallas, Texas 75235 in room 3.120. Additional planned components (a high-end immersive driving/task simulator and an eight camera human motion capture and analysis system) were also constructed and are located at the University of Texas at Arlington's Human Performance Institute, Nedderman Hall, Room 241.

The pilot study (Task #1b.) has not been completed. The protocol was approved by the University of Texas Institutional Review Board in June 2007. The protocol remains (as of September 2008) at the U.S. Army Medical Research and Material Command's Office of Research Protections, Human Research Protections

Office and we have not received approval from that Office. Upon its approval by USAMRMC HRPO this pilot study will be performed and completed.

We applied for three DOD proposals during the period of this grant that would have utilized this multimodal laboratory (see list below). None of these proposals was funded.

: 1. DOD Opportunity ID: W81XWH-07-PTSD-MRC (Consortium); Proposal Title: The Neurobiology of PTSD: Risk Factors, Diagnosis, Course and Treatment (Functional Study of Biological Molecules)(Robbie Greene)

2. DOD Opportunity ID: W81XWH-07-CC-CSS (PTSD/TBI Clinical Consortium-Study Site); Proposal Title: "North Texas PTSD/TBI Clinical Research Center." (Ramon Diaz-Arrastia)

3. DOD Opportunity ID: W81XWH-07-PTSD-IIRA-INT (Investigator Initiated); Proposal Title: Repetitive Transcranial Magnetic Stimulation (rTMS) to Reduce Over-Arousal to Combat-Related Emotional Stimuli (John Hart, Jr.)

Dr. Kondraske was invited by Lt. Col. Valerie Martindale, AFHSIO to make two presentations related to the broad project objectives for a panel session on Human Performance Optimization at the 2008 Aerospace Medical Association meeting in May:

Kondraske, G.V. (2008) General systems performance theory and the elemental resource model for human performance. *Aviation, Space, and Environmental Medicine*, 79(3): 250. (presented at the 79th Annual Scientific Meeting of the Aerospace Medical Association, Boston, May 13, 2008).

Kondraske, G.V. (2008) General systems performance theory and human performance: some experimental results. *Aviation, Space, and Environmental Medicine*, 79(3): 250-251. (presented at the 79th Annual Scientific Meeting of the Aerospace Medical Association, Boston, May 13, 2008).

Copies of abstracts are attached.

CONCLUSIONS

In summary, the assimilation of this unique multimodal human cognitive-motor assessment laboratory is a significant accomplishment and will allow for numerous innovative studies. We plan to perform the pilot study proposed and have multiple other planned studies for the future, including already targeted grant proposals.

Unfortunately, the inability to receive HRPO approval of the proposed pilot study, even after UT IRB approval, before the ending of the granting period limits the implications from this project.

APPENDICES

File {dod rTMSirb.pdf} which is the University of Texas consent form with IRB approval of the pilot project proposed.

Files {P08_01_AN.pdf} and {P08_02_AN.pdf} are abstracts of presentations made by Dr. Kondraske

Project #2 Ubiquitination of Proteins in the Normal and Pathological Brain

INTRODUCTION

Hypothesis: αSpI ubiquitinates itself and binding partners in neurons, skeletal and cardiac muscle. Polyubiquitination leads to turnover by the ubiquitin proteasome system, while monoubiquitination regulates protein-protein interactions within the membrane skeleton. Diminishing spectrin E2/E3 activity within neurons and cardiac muscle fibers will lead to diminished ubiquitination, accumulation of damaged protein, and a membrane skeleton that cannot readily disassemble or reassemble.

Task 1. Create and phenotype single mutant mice (C2068A).
Task 2. Utilize these mutant mice versus wild type for determination of the targets of spectrin's E2/E3 activity in brain, muscle and RBCs.
Task 3. Localize the precise ubiquitination sites on all targets by performing tandem mass spectrometry on tryptic digests and searching for the gly-gly ubiquitin remnant (m/z=114.1).

Body

Task 1. Create and phenotype single mutant mice (C2068A).
We have obtained 2 BAC clones from a C57 strain library, both of which were predicted to contain a large portion of the Spna1 gene as determined using the ENSEMBL Genome Browser (Clones RP23-343E12 and RP23-252F11). Using oligonucleotides based upon the sequence of Exon 43 (numbered using 51 exons in the total gene), we attempted to generate a probe of ~500bp containing the entire 297 bp of exon 43 and flanking DNA
to serve as a probe to screen the CHORI 129 BAC library filters. Only clone 252F11 was able to give the appropriate sized fragment (Figure 1) and this fragment was verified by DNA sequencing. This DNA was labeled with 32P and used to screen the 129 BAC filters, producing 12 strong positive hybridizing signals (see Figure 2), 6 of which were ordered from CHORI. We then used a PCR assay to.evaluate which of the 129 BACs contained exon 43, (region of interest) and found 2 clones (CHORI129 -599I19 and CHORI129-99P17) that gave the 250 bp product. We have used Clone 99P17 exclusively to generate PCR products for the arms of the mutant vector, to be constructed as shown in Figure 3. Both arms (5' – intron 42 to intron 43; 3' - intron 43 to intron 45) were

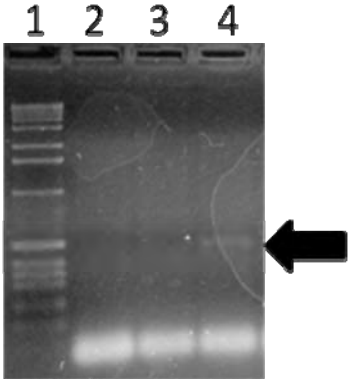


Figure 1. PCR generation of the probe fragment containingSpna1 exon 43 segment. Oligonucleotides that were 5' and 3' to the 297 bp exon43 were used to amplify a segment of DNA from the C57 BAC clones R23-343E12 (lane 3) or RP23-252F11 (lane 4). Lane 1 contains a Kb DNA standard and Lane 2 shows the result obtained from a reaction with no added DNA. The arrow shows the amplified fragment of 527 bp in size

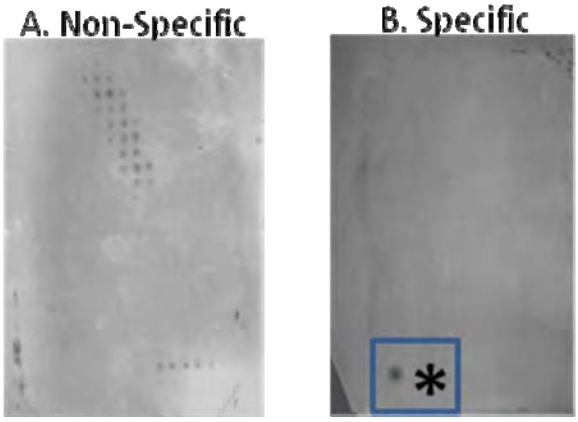


Figure 2. Sample filter hybridizations of CHORI 129 library filters. Filters from CHORI containing the 129 BAC library were hybridized with the fragment of DNA containing Snpa1 exon 43. Segment A shows non-specific hybridization signals with a specific hybridization signal shown in B. This signal correspondsd to clone CHORI129-99P17 which was used in further experiments.

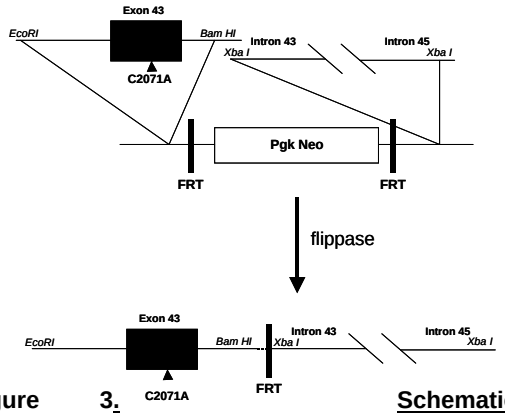


Figure 3. Schematic of mutant vector construction. A diagram of how the design of Spna1 mutant vector C2071A Targeted Allele **to construct the Cys to Ala change is shown.**

PCR amplified and cloned into PCRScript (Stratagene). We are now determining the sequence for the cloned fragments, which will allow further construction of the mutant vector. As illustrated in Figure 3 we will make the mutant sequence within the 5' arm segment by PCR to generate the C to A mutant. We have chosen a method that will generate the amino acid mutant and change the DNA sequence to mutate a MunI restriction site. This site will be utilized for validation of the mutant DNA sequence following insertion into the mouse ES cell genome by homologous recombination. Once we construct the mutant DNA vector, we will utilize the Texas A&M GEMcore (Genetically Engineered Mouse core) to place it into the AB2.2 ES cells and to inject the appropriate ES cell lines into blastocysts. Dr. Zimmer is Co-Director of this facility along with Dr. Danna Zimmer, an IBMST faculty member. The mice, once created will be bred and used as outlined to determine the effect of changing the C ^{2071} equivalent in mouse on the general phenotype as well as in specific cellular contexts. We have integrated the purchased equipment into the project. The Fuji phosphoimager has been used in the screening of the BAC libraries and the Fuji ChemiDoc has been extensively used to evaluate agarose gels for the correct PCR fragments and vector construction. This equipment will be utilized to facilitate construction of this and future mutant mice strains.
Task #2 and Task #3 Can be performed once the mutant mice are prepared.

KEY RESEARCH ACCOMPLISHMENTS

We have isolated clones that contain the region of interest of alpha spectrin (exon 43). We have used Clone 99P17 to generate PCR products for the arms of the mutant vector. Once we construct the mutant DNA vector, we will utilize the Texas A&M GEMcore (Genetically Engineered Mouse core) to place it into the AB2.2 ES cells and to inject the appropriate ES cell lines into blastocysts. The mice, once created will be bred and used as outlined to determine the effect of changing the C ^{2071} equivalent in mouse on the general phenotype as well as in specific cellular contexts. We have integrated the purchased equipment into the project. The Fuji phosphoimager has been used in the screening of the BAC libraries and the Fuji ChemiDoc has been extensively used to evaluate agarose gels for the correct PCR fragments and vector construction. This equipment will be utilized to facilitate construction of this and future mutant mice strains.

REPORTABLE OUTCOMES

We have isolated and characterized the necessary exon 43 clones of alpha spectrin and produced the arms for the mutant vectors. We are now prepared to produce the mutant mice required for aims 2 and 3 of this study.

CONCLUSIONS

All of the molecular biology has been performed to produce the alpha spectrin mutant mice and necessary equipment has been purchased that is necessary to pursue Tasks 2 an3.

APPENDICES

None.

Project #3: Carbon Nanotubes and Cancer

Introduction

Despite the success of current treatments for several types of cancer, all known treatments have major limitations. To decrease nonspecific toxic effects, targeted therapies are being developed and some have already been approved by the FDA for use in humans. These use both small molecules that target specific intracellular pathways in tumor cells and monoclonal antibodies that target cell surface molecules on tumor cells. However, these targeted agents are frequently cytostatic, not cytotoxic, and they are often given in combination with chemotherapy in an effort to both lower the dose of chemotherapy required and to achieve synergistic effects. An approach to chemotherapy that combines both targeting and multiple ways to deliver a lethal blow to tumor cells would help solve some the current problems. The use of carbon nanotubes (CNTs) in cancer chemotherapy may support such a targeted multi-modal strategy for cancer chemotherapy. There are three objectives in this proposal. First, to couple monoclonal antibodies (MAbs) to CNTs. Second, to test the targeting and uptake of CNTs to cells via the MAb in model tumor cell culture models. Third, to assess the thermal ablation of the model tumor cells mediated by the CNTs upon irradiation with near infra-red light.

Body

Task 1. To couple monoclonal antibodies (MAbs) to single-walled carbon nanotubes (CNTs) (Months 1-4). The MAbs we will use in pilot studies are anti-HER2 antibodies that bind breast adenocarcinoma cells and J591 antibodies that bind prostate adenocarcinoma cells.

(After the project began, we had the opportunity to collaborate with Dr. Ellen Vitetta and colleagues at UT Southwestern who had developed MAbs that bind Daudi cells, a lymphoma cell line. In what follows, we substituted work on the Daudi cell tumor model system for work with the prostate adenocarcinoma model system, and have incorporated the results into the progress report. This includes the addition of an item c under Task 1, which describes how Daudi-reactive MAbs were attached to SWNTs)

a. Approach one, to very lightly oxidize the CNTs to introduce carboxyl groups and then to employ standard chemical reactions to couple the MAbs to the carboxyl groups (Months 1-3).
b. Approach two, to attach the MAbs to a derivative of a cyclic peptide that we have shown in previous work encircles the CNTs, rendering them water soluble (Months 2-4).
c. Approach three, to attach biotin to SWNTs using a commercially available biotinylated lipid dispersant that can bind MAbs that have been covalently conjugated to a streptavidin derivative, Neutravidin. (Months 2-4)

Each of these approaches is described individually in the following subsections.

Task 1a, approach one, to very lightly oxidize the CNTs to introduce carboxyl groups and then to employ standard chemical reactions to couple the MAbs to the carboxyl groups (Months 1-3).

Progress task 1a:

Oxidation of Single-walled CNTs (SWNTs). Raw HiPco SWNTs were dispersed via sonication in an aqueous solution of Triton X-100 prior to chemical oxidation with aqueous nitric acid. Carboxylic acid groups are known to be generated at the end caps and along the sidewalls of the SWNTs under these conditions. It is important

in this approach to measure the extent of carboxylation to ensure that the optical properties of the SWNTs are not destroyed by the over-carboxylation. We measured the extent of carboxylation as a function of oxidation time and determined that a time of 24h introduced 11% carboxylation. At this extent of carboxylation, the SWNTs retained their essential optical properties. Complete details of this work are in a Masters Thesis by Pooja Bajaj (Bajaj, 2008).

The overall strategy for attaching MAbs to SWNTs is to biotinylate the SWNTs at carboxylation sites, then employ various ways to couple the MAbs to the SWNTs using the very high affinity of NeutrAvidin (a streptavidin derivative) for biotin to form MAb/SWNT complexes. Biotin LC-PEO-amine (commercially available) was covalently bound to SWNTs through surface carboxyl groups created under mild acid oxidation conditions. These carboxylic acid groups provided the necessary chemical anchor to covalently couple biotin LC-PEO-amine by the ethylene diamine carbodiimide (EDC)-mediated reaction in MES buffer resulting in amide bond formation. As described in our recent publication (Chakravarty et al., 2008) biotin on SWNTs can be used in strategies to target MAb-Neutravidin constructs to cancer cells, diagramed in Figure 1.

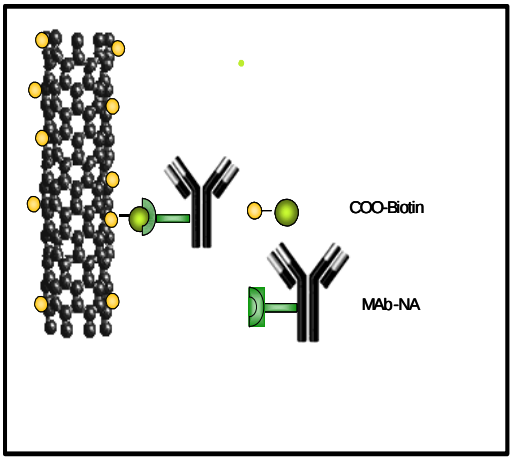


Figure 1. Noncovalent attachment of neutravidin-conjugated MAbs to biotinylated SWNTs. Biotin LC-PEO-amine was covalently bound to SWNTs through surface carboxyl groups created through mild acid oxidation. The biotin can then bind MAbs that have been conjugated to NeutrAvidin.

Characterization of carboxylated SWNTs covalently coupled to Biotin LC-PEO-amine. Carboxylated and biotinylated SWNTs were visualized by atomic force microscopy (AFM) (Figure 2), transmission electron microcsopy (TEM) (Figure 3), their optical properties measured using UV-Vis-NIR spectroscopy (Figure 4), and the chemical coupling of biotin to SWNT surface carboxyl groups assessed by Fourier Transform Infrared Spectroscopy (FTIR) (Figure 5). AFM images of carboxylated SWNTs dispersed in water (Figure 2a) demonstrate a good dispersion of individual and small bundles of SWNTs. Upon biotinylation, features suggesting covalently coupled biotin were observed along the length of the SWNTs as indicated with the arrows (Figure 2b).

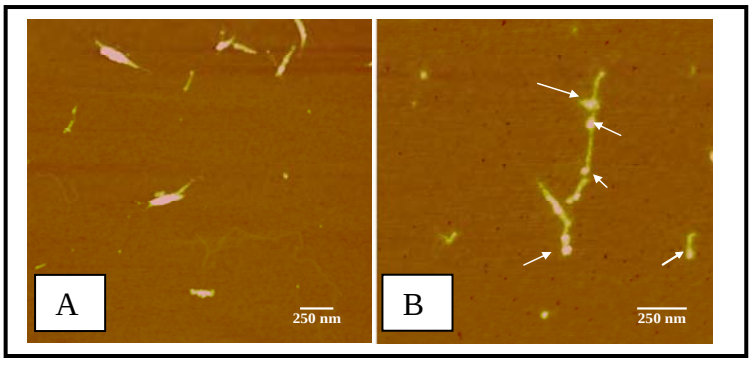
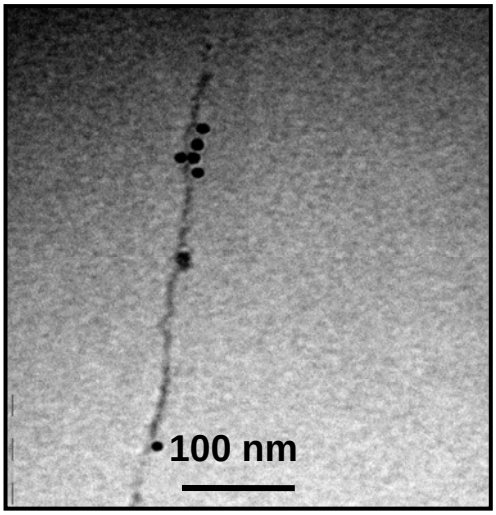


Figure 2. AFM images of SWNT dispersions. The SWNTs were functionalized with carboxylic acid groups by a mild nitric acid reflux. After neutralization, they were resuspended in distilled water by sonicating for 10 min and centrifuging at 16,000 x g for 10 min. The supernatant was collected for analysis. The carboxylic acid modified SWNTs were covalently coupled to Biotin-LC-PEO-amine via an EDC mediated reaction in MES buffer. **(a)** AFM image of carboxylated SWNTs illustrating a good dispersion, and **(b)** AFM image of biotinylated SWNTs showing covalently coupled biotin moieties at carboxyl functionalized sites (arrows point to biotinylated sites).

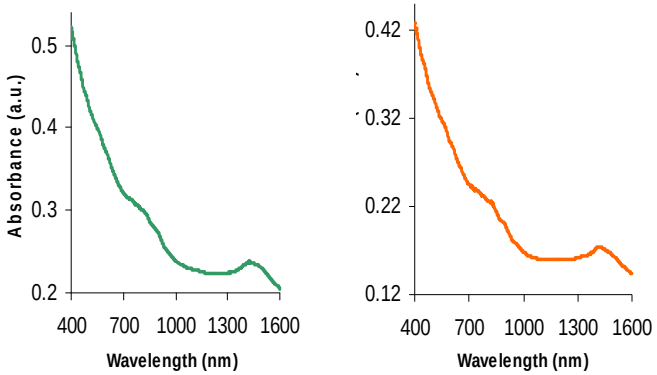
To more directly assess the presence of biotin on SWNTs, biotin was detected by TEM using streptavidin-labelled gold (SA-Au). As seen in Figure 4, SA-Au bound to discrete sites only present on the SWNTs, suggesting that biotin was indeed attached to the SWNTs.

Figure 3 Binding of SA-Au particles to biotinylated SWNTs assessed by TEM. 100 µL of biotinylated SWNT dispersion was incubated with 5 µL of 1:50 SA-Au solution, vortexed, and reacted for 12h. The sample was ultracentrifuged to pellet the SWNTs and separate them from unbound SA-Au particles. The pellet was resuspend in DI water and 5 µL dropped on the grid. Excess water was wicked away and the samples dried for TEM.

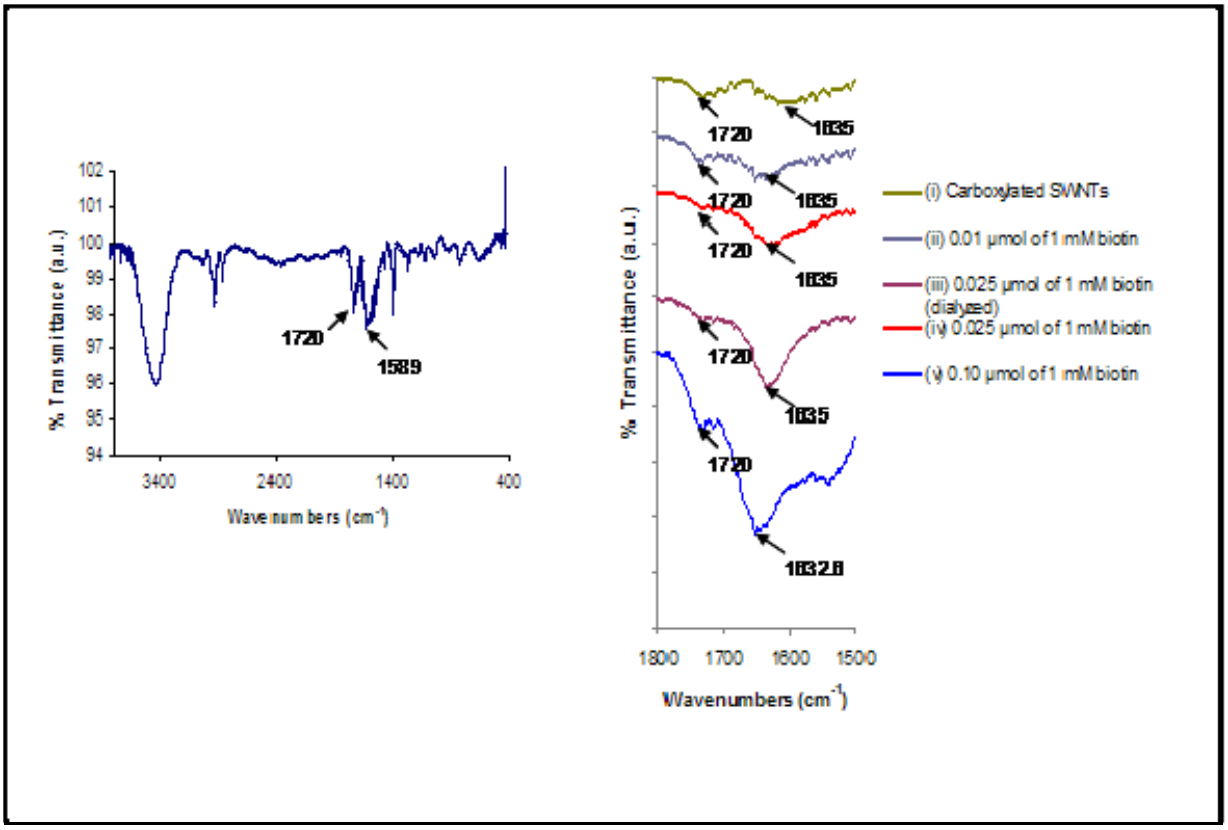


UV-Vis-NIR spectra of carboxylated SWNTs in MES buffer show the presence of van Hove singularities (Figure 4a) that are retained after biotinylation of the carboxyl sites (Figure 4b). This demonstrates that the carboxylated and biotinylated SWNTs retain the ability to absorb NIR, necessary for heating of SWNTs exposed to NIR.

Figure 4. Uv-Vis-NIR spectra of **(a)** carboxylated SWNTs in MES buffer, and **(b)** biotinylated SWNTs redispersed in DI water.



FTIR spectra acquired from biotinylated SWNTs were compared to those of carboxylated SWNTs in order to confirm the covalent coupling, through amide bond formation, of Biotin-LC-PEO-amine to the SWNT surface carboxyl groups. FTIR spectra of carboxylated SWNTs (Figure 5 left and 5 right (i)) exhibit peaks at 1720 cm^{-1} and 1589 cm^{-1} characteristic of the carbonyl stretch (C=O) and C=C stretch, respectively. As biotinylation progresses (Figure 5b (ii) → (v)), the peak for the carbonyl stretch associated with the carboxylic acid (1720 cm^{-1}) decreases and the peak associated with the amide (1635 cm^{-1}) increases.



SUMMARY, TASK 1A: We have lightly carboxylated SWNTs and attached biotin to them. The biotin can now be exploited to attach any MAb that can be coupled directly or indirectly to the biotin for targeting of SWNTs to epitopes on cells that the MAb binds.

Figure 5. FTIR spectra of SWNTs. (Left) Carboxylated SWNTs showing the carbonyl stretch (C=O) at 1720 cm^{-1} and the C=C stretch at 1589 cm^{-1}. (Right) SWNTs showing changes in the 1500-1800 cm^{-1} region during biotinylation. In order: (i) carboxylated SWNTs refluxed in nitric acid; (ii) to (v) biotinylated SWNTs with increasing biotin concentration in the reaction system. The carbonyl peak at 1720 cm^{-1}, owing to the carboxylic acid, decreases in intensity and a peak originating at 1635 cm^{-1}, attributed to the carbonyl (C=O) in the amide, simultaneously increases.

Task 1b, approach two, to attach the MAbs to a derivative of a cyclic peptide that we have shown in previous work encircles the CNTs, rendering them water soluble (Months 2-4).

Progress task 1b:

We previously described cyclic peptides that encircle SWNTs, make them water soluble, and provide a platform for attaching ligands to the SWNTs (Ortiz-Acevedo et al., 2005) A model of the peptide wrapping a SWNT is shown in Figure 6.

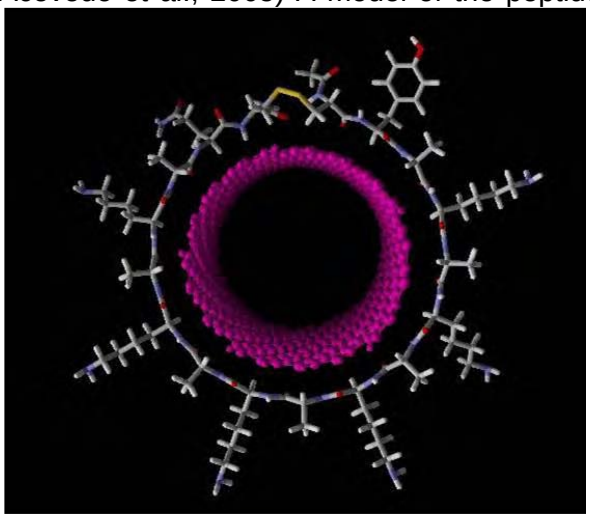


Figure 6. Energy minimized model of cyclic peptide RC7-Cys encircling a SWNT. The pepide contains alternating D-Alanine and L-Lysine to produce curvature and Cysteine residues at the each end to covalently close around the SWNTs. The entire peptide sequence is:

C-Y-^{D}A-K-^{D}A-K-^{D}A-K-^{D}A-K-^{D}A-K-^{D}A-K-^{D}A-Q-C

The strategy for attaching SWNTs to MAbs with this approach is to incorporate biotin into the peptide, and use biotin/NeutrAvidin interactions to couple the SWNTs to MAbs, similar to the approach outlined in Figure 1. Since the peptides are chemically synthesized in house, we can incorporate functionalities like biotin at any location desired in the peptide. The scheme we used to put biotin on a single lysine residue of the cyclic peptide is outlined in Figure 7. The peptide was purified and analyzed by mass spectrometry to verify that it was the correct structure.

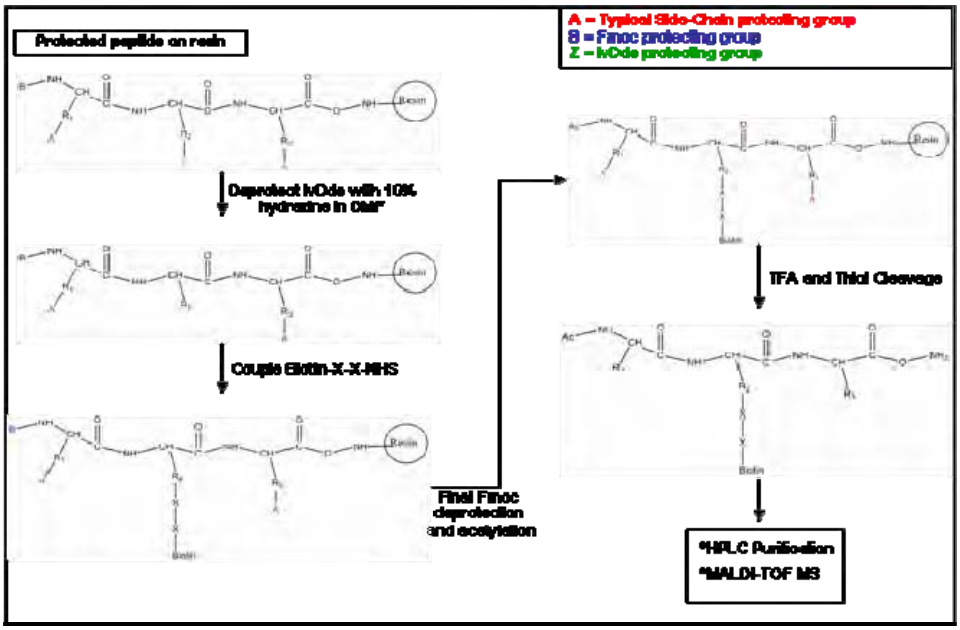
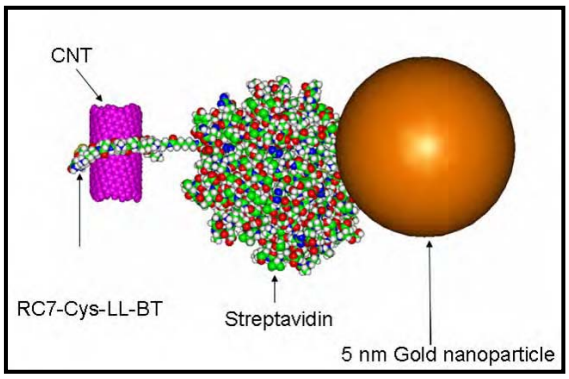


Figure 7. Synthesis, purification, and verification of RC7-Cys containing a single biotin residue.

To confirm that biotin was present, we did TEM of SWNTs wrapped with the biotinylated peptide using streptavidin-gold conjugates to locate biotin on the SWNTs. A schematic of the approach is presented in Figure 8. The TEM images are shown in Figure 9. Gold particles are readily detected along the length of SWNTs wrapped with cyclic peptide that contains biotin, and the greater the percent of biotinylated peptide (compared to non-biotinylated peptide) used to wrap the SWNTs, the greater is the gold labeling (Figure 9).



- Dispersion diluted 1:10 with water
- Gold reagent diluted 1:2 in water
  - Streptavidin labeled with 5 nm gold nanoparticles
- Labeled CNTs pelleted by centrifugation and resuspended in water.
- Sample dropped onto Formvar Only TEM grid and wicked away after 1 min.
- Sample stained with 1% Urinyl Acetate for 30 seconds.

Figure 8. Protocol for TEM of gold-labeled SWNTs wrapped with RC7-Cys-biotin.

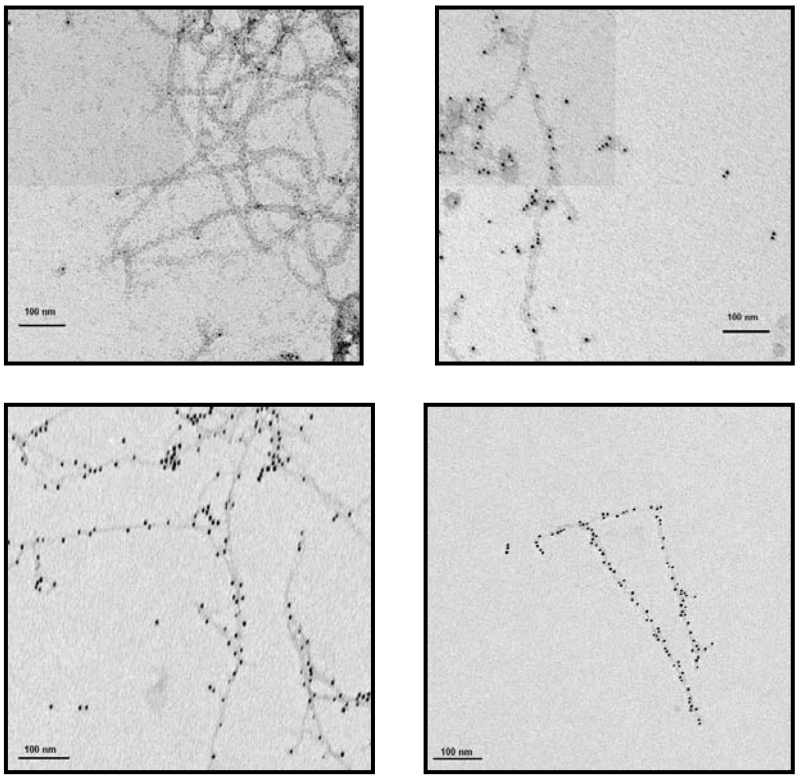


Figure 9. TEM images of SWNTs wrapped with different ratios of biotinylated cyclic peptides to non-biotinylated peptides.

Top left, 0% biotinylated;
Top right, 10% biotinylated;
Lower left; 50% biotinylated;
Lower right, 100% biotinlyated.

The biotin residues are identified by the binding of 5nm gold particles conjugated to streptavidin.

SUMMARY, TASK 1B. The cyclic peptide RC7-Cys with a single biotin on a lysine residue was synthesized, purified, and the structure verified by mass spectrometry. The material was used to disperse SWNTs and the presence of biotin on the SWNTs was verified by TEM. During this work, we discovered that the cyclic peptide dispersed SWNTs aggregated in cell culture medium. We think the reason for this is that the relatively high salt concentration in medium shields the positive charges on lysine residues in the peptide, allowing close approach of the dispersed SWNTs in salt, resulting in aggregation. This aggregation precluded tests of cell binding and uptake in tasks 2 and 3. We are presently redesigning the cyclic peptide to remove most of the lysine residues, which should produce a dispersant that is less sensitive to aggregation by salt.

Task 1c, approach three, to attach biotin to CNTs and use MAbs coupled to Neutravidin to direct the biotinylated CNTs to tumor cells.

Progress task 1c:

This approach uses a biotinylated lipid molecule that non-covalently binds to and disperses SWNTs. The preparation of the biotinylated SWNTs and coupling of the material to MAbs conjugated NeutrAvidin are described in a manuscript published in June, 2008, and attached as Appendix A (Chakravarty et al, 2008).

SUMMARY, TASK 1C: Both tumor specific and control MAbs have been attached to SWNTs (see appendix A). As described next in tasks 2 and 3, SWNTs have been targeted to tumor cells with this system, and thermally ablated them with NIR light.

Task 2. To test the targeting of CNTs to cells via the MAb in tumor cell culture models (Months 5-9): We will focus on BT-474 breast adenocarcinoma cells and LNCaP prostate adenocarcinoma cells:

a. Test the binding of CNT-MAbs to the surface of cells (Months 5-7).
b. Assess whether the binding of CNT-MAbs to cells is specific (Months 5-7).

c. Measure the internalization of CNT-MAbs by cells and determine their subcellular location. (Months 7-9)

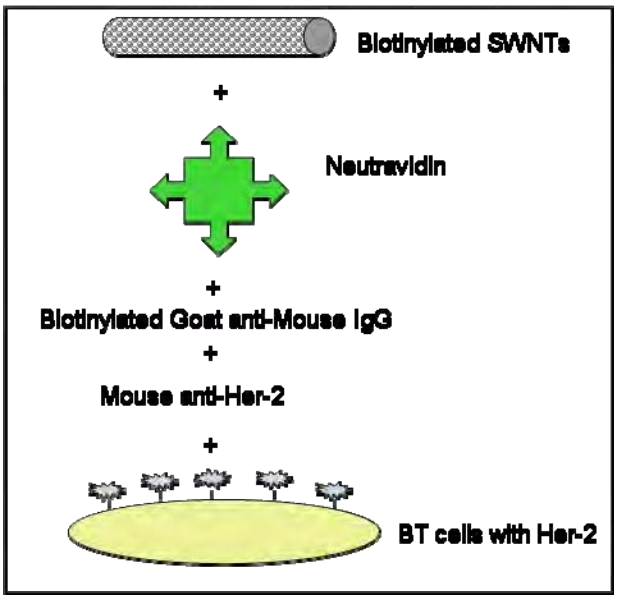
Of the three methods described in task 1 for attaching MAbs to SWNTs, two were carried forth into task 2: the carboxylation and direct addition of biotin to SWNTs (approach 1) and the use of biotinylated lipids to disperse SWNTs (approach 3). The cyclic peptide method (approach 2) was not carried into task 2 due to unanticipated aggregation problems, as described in the previous section.

Task 2a, to test the binding of CNT-MAbs to the surface of cells (Months 5-7) and task 2b, to assess whether the binding of CNT-MAbs to cells is specific.

Progress with carboxylated and biotinylated SWNTs (prepared in task 1a).

The method for targeting biotinylated SWNTs to BT-474 cells is summarized in Figure 10. A reagent sandwich approach is used in the targeting and relies on the fact that NeutrAvidin has 4 binding sites and can bind both a biotinylated MAb on the cell surface and biotinylated SWNTs. To directly detect SWNTs associated with the cells, we exploited the distinctive Raman signature of SWNTs (termed the "G" band) that can be measured using a confocal Raman microscope. The laser is aimed at the cell of interest and the Raman spectrum is recorded. As a control, the laser is also aimed at a location on the dish where there are no cells and the spectrum is recorded. The area under the G band peak is used as an estimation of the SWNT amount.

Figure 10. Method for targeting and binding SWNT/biotin complexes to breast BT-474 adenocarcinoma cells. These cells overexpress the Her-2 protein and we use anti-Her-2 MAbs to target the SWNTs to the cells. We obtained our anti-Her-2 (Her-66) courtesy of Dr. Ellen Vitetta (UTSW Medical Center). The cells were fixed, incubated with anti-her-2, washed, incubated with biotinylated goat-anti-mouse IgG, washed, and NeutrAvidin was added.



The general method for sample preparation was as follows: BT-474 cells were plated at $2x10^4$ per well on 12mm coverslips in 4 well cell culture dishes and grown at 37^o for six days in media recommended by ATCC for these cells and supplemented with 10% FBS and antibiotics. The cells were washed 3 times in PBS, and then fixed with cold 4% paraformaldehyde at room temperature for 15 min., and washed again 3 times. BSA (10mg/ml) was added and the cells were rocked for 10 min to block non-specific sites. The primary antibody, mouse anti-Her 2 antibody (Her 66 from E. Vitetta 1.47 mg/ml) was diluted either 1:1000 or 1:10,000 in BSA (10 mg/ml in PBS) and 15 ul was placed on each coverslip except for the control well. The antibody was allowed to bind for 45 min at room temp then the antibody was removed by washing 3 times with PBS and BSA was added as previously and reacted for 10 min. The secondary antibody, goat anti mouse IgG conjugated with biotin (Pierce 318000) at a conc. of 1.1 mg/ml, was diluted 1:1000 or 1:5000 in BSA as above and 15 ul was placed on each coverslip. The antibody was allowed to bind for 30 min, and removed by washing 3 times with PBS. BSA was added for 10 min then the coverslips were washed once with PBS. The NeutrAvidin conjugated with FITC (Pierce 31006) at a conc.of 5.5 mg/ml in PBS was diluted 1:5000 in PBS and 15 ul was placed on each coverslip. The NeutrAvidin was allowed to bind for 35 min and removed by washing 3 times with PBS. Biotinylated SWNTs that had been dialyzed (15ul) was added to each coverslip and allowed to bind

for 30 min. The excess material was removed by washing with PBS. The coverslips were mounted onto a slide with Fluoromount G.

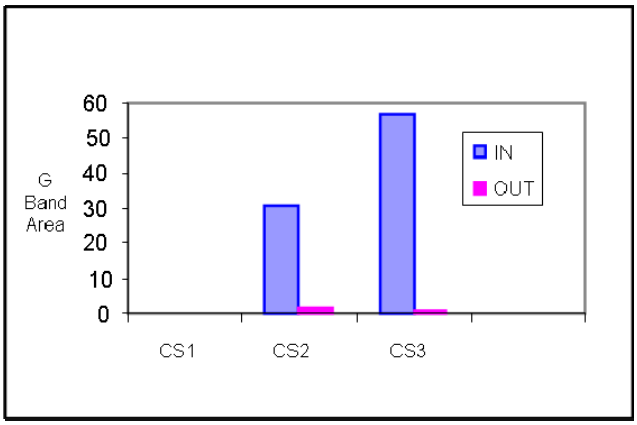
After optimizing the conditions and concentrations of reagents, specific details of the cell samples we studied are summarized in Table 1. Sample CS1 is a control with no primary antibody; samples CS2 and CS3 had all reagents, but differed in the type of NeutrAvidin used (NA alone or NA conjugated to FITC).

4 Samples:	CS1	CS2	CS3
Primary AB	none	Yes (1/1000)	Yes (1/1000)
Secondary AB	Yes (1/1000)	Yes (1/1000)	Yes (1/1000)
NA or FITC- NA	FITC-NA (1/2500)	NA (1/2500)	FITC-NA (1/2500)
Biotin-SWNT	15ul	15ul	15ul

Table 1. Reagents added to samples for targeting biotinylated SWNTs to BT-474 cells.

The area under the Raman G bands for the different samples of Table 1 are shown in Figure 11 when the laser was either pointed within a cell boundary (in) or outside a cell boundary (out). Sample CS1 had no primary antibody, and no signal is seen. Samples CS2 and CS3 had all reagents, but differed in the type of NeutrAvidin used. Both showed the presence of the SWNT G band. The fact that there was no signal in cells where only the primary antibody was missing is evidence of specific binding.

Figure 11. Raman signal when laser is aimed at a cell (blue) or outside a cell (pink). Sample CS1 had no primary antibody, and no signal is seen. Sample CS2 and CS3 had all reagents, but differed in the type of Neutravidin used. Both showed the presence of the SWNT G band. Sample CS4 had a reduced amount of Neutravidin and the G band signal declined.



Progress with SWNTs dispersed by a biotinylated lipid prepared in task 1c.

SWNTs dispersed with a biotinylated lipid were targeted to Daudi cells by attaching MAbs to NeutrAvidin and using the interaction of NeutraAvidin with biotin on the SWNTs to form a complex that was then incubated with Daudi cells. Details of the binding methods and the specificity of the method are presented in published manuscript, attached as Appendix A (Chakravarty et al., 2008).

Progress with task 2c, to measure the internalization of CNT-MAbs by cells and determine their subcellular location. (Months 7-9)

To quantify the uptake and subcellular location SWNTs, we had to develop a new method for measuring cell-associated SWNTs that was sensitive enough to detect the low levels of material in cells. To develop this

method, we used SWNTs dispersed in culture medium that were not targeted to cells because the targeting systems are very expensive due to the cost of MAbs. Also, we have had previous experience in different model systems on the uptake by cells of non-targeted SWNTs via fluid-phase endocytosis (Chin et al., 2007; Yehia et al., 2007). With the non-targeted model system we successfully developed a new approach to quantifying SWNTs in cells and tissues that involved extracting the SWNTs with hot SDS followed by gel electrophoresis that concentrated the SWNTs at the buffer/stacking gel interface. The intensity of the band formed at the interface is proportional to the amount of SWNTs extracted and we can measure as little as 2 ng SWNTs in this system. The method was deemed so successful and potentially useful that the patent attorney for the university advised us to patent the method. Appendix B is the disclosure of the patent to the university and will be the basis for preparation of the patent document. The disclosure presents the method and results in detail (Appendix B). Now that this method is developed, we can apply it to measure the binding, internalization, and subcellular location of SWNTs targeted to cells with MAbs in future work.

SUMMARY OF TASKs 2A, 2B , and 2C:

Carboxylated/biotinylated SWNTs were targeted to fixed BT-474 cells by the anti-Her-2 antibody using the multicomponent assay. Evidence of specificity was that leaving out the primary antibody did not produce the signature Raman G band associated with SWNTs.
With the Daudi cell system, the SWNT/biotin/MAb complex was specifically targeted to cells that had the correct antigen for the antibody used. Note that two different antibodies were employed to target SWNTs and that the SWNTs/biotin/complex only bound to cells that were known to react with appropriate antibody (Appendix A).

Task 3. To assess the thermal ablation of the tumor cells in the model cell systems upon irradiation with near infra-red light (NIR) (Months 9-12):

a. To load cells with different amounts of CNT-MAbs, as determined in Task 2 and expose them to NIR (Months 9-10)
b. To assess the viability of cells exposed to CNT-MAbs after exposure to NIR (Months 9-10)
c. To optimize conditions for loading cells with CNT-MAbs and exposing the cells to NIR to maximize specific ablation of tumor cells (Months 10-12).

Progress in Task 3, to assess the thermal ablation of the tumor cells in the model cell systems upon irradiation with near infra-red light (NIR) (Months 9-12)

Summary: Of the model systems explored in this work, the Daudi cell lymphoma cancer model was the best developed for the final task, targeted photo-thermal ablation of cancer cells in culture. The work was successful and resulted in a publication (Chakravarty et al., 2008). Details of the work are given in the manuscript attached as Appendix A.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have optimized a nitric acid reflux process that produces carboxylated SWNTs that form stable dispersions in DI water while maintaining their optical absorption properties.
2. Carboxylated SWNTs have been chemically coupled to Biotin-LC-PEO amine as evidenced by AFM, TEM, and FTIR spectroscopic studies.
3. Biotiylated SWNTs have been targeted to breast adenocarinoma cells using anti-her2 MAb and NeutraAvidin to bridge between the MAb and the biotinylated SWNTs.
4. Cyclic peptides containing biotin have beensynthesized and used to wrap SWNTs. The presence of biotin on the SWNTs was demonstrated by streptavidin-mediated binding of 5 nm gold particles using TEM.
5. We developed a new approach to extract and quantify SWNTs in cells and tissues that can detect as little as 2-5 ng SWNTs in a 50 µl sample.

6. Using the Daudi cell system, in collaboration with Ellen Vitetta at UT Southwestern Medical School, we coupled a form of biotinylated SWNTs to MAb NeutrAvidin conjugates, targeted these specifically to cells that react with the targeting MAb, and thermally ablated only cells that bound the correct MAb/SWNT complex.

REPORTABLE OUTCOMES:

1. An abstract for an oral presentation was submitted to the Microscopy and Microanalysis 2008 conference to be held in Albuquerque, August 2008, and the presentation was made.
2. A manuscript and M.S. Chemistry thesis are in preparation that details the time-dependent study to prepare carboxylated SWNTs via mild acid oxidation.
3. A patent disclosure was filed at UT Dallas describing the method for extracting and quantifying SWNTs from cells and tissues (Appendix B). A manuscript on this method is in preparation.
4. SWNT/MAb complexes have been specifically targeted to Daudi cells (and control cells), and the targeted cells have been efficiently killed with NIR. This work is now published (Chakravarty et al., 2008) (Appendix B).

CONCLUSION

SWNTs can be targeted to cancer cells with MAbs and the cancer cells can be eliminated by thermal ablation upon exposure to NIR light. This non-invasive and selective approach to cancer chemotherapy is viable and is now being expanded into animal studies. So what? If this method is successful in eliminating human tumors in mice it may prove to be a strong weapon in the war against cancer.

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Project #4: Advanced DNA Microarray Technologies based on Homologous Strand Exchange and Magnetic Nanomanipulation

Introduction

Homologous base-pairing interactions are the physical basis for many nucleic-acid-based diagnostic and forensic techniques. Within the last decade, adaptation of nucleic-acid-hybridization methods to high-throughput technologies such as microarray analysis has dramatically advanced the identification of complex disease states, detection of mutations and polymorphisms, discovery of new drugs, and detection of pathogens. It is generally agreed that fine-structure mapping of DNA rearrangements such as those in leukemia and lymphoma requires stringent hybridization technologies for DNAs in the size range of 100 bp to several kbp. Present high-throughput technologies are restricted to targeting relatively short nucleic-acid-sequence motifs (on the order of 50 base pairs) because longer sequences can form relatively stable hybrids even in the presence of base mismatches of significant length or insertions/deletions on either nucleic-acid strand. To overcome this limitation we will develop a novel microarray-based hybridization technology that exploits the homologous pairing of DNA strands driven by the RecA protein of *E. coli*. This protein-induced strand-association reaction is highly specific, chemically and mechanically reversible, and amenable to detection by surface-plasmon-resonance techniques. Using magnetic torque-induced dissocation of RecA-DNA complexes conjugated to magnetic nanoparticles, we will establish the use of magnetic-field-dependent strand-association isotherms as a sensitive method for detecting hybridization of motifs up to 300 bp.

Body

The original proposal contained three technical objectives:

- **i.** Establish proof of principle that RecA-mediated strand exchange can be used to identify and discriminate DNA-sequence alterations associated with the molecular etiology of human cancers

- **ii.** Detect the formation and dissociation of RecA-DNA synaptic complexes and quantify complex stability using surface-plasmon resonance spectroscopy

- **iii.** Develop methodologies for the attachment of probe DNA duplexes to surfaces and magnetic nanoparticles

In response to reviewer concerns about applications of the technology to large-scale screening, we re-prioritized these objectives to focus on (**ii.**) at the expense of (**i.**) and (**iii.**). In particular, we undertook the development of a novel surface-plasmon-resonance-imaging instrument (SPRI) design. To our knowledge, this is the only current instrument housing a permanent magnetic field. Previous results that bear on (**i.**) are described below, followed by progress reports on (**ii.**) and (**iii.**) over the 12-month period 8/07 – 8/08.

Task i.

RecA-mediated strand exchange. Pairing of DNA strands mediated by the *E. coli* RecA protein and its homologs in higher organisms is an essential step in general DNA recombination, an essential biological process for correcting damage to a cell's genetic code. As shown in **Fig. 1**, the RecA strand-exchange protein binds to single-stranded DNA (ssDNA) in the presence of ATP, or its slowly hydrolyzable form, ATPγS (adenosine 5'-O-(3-thio)triphosphate), to form a right-handed nucleoprotein filament. This nucleoprotein filament subsequently searches for homologous sequences on duplex DNA (dsDNA), followed by pairing of the incoming strand and its complement. A triple-stranded intermediate is formed consisting of a new DNA structure in which the incoming single strand is paired with the complementary strand from the original duplex and the nascent displaced strand is wound around the newly formed duplex region. For DNAs 100 bp to 1 kbp, RecA-dependent homologous pairing has much higher specificity than normal Watson-Crick base pairing; local disruptions of homology greater than 3 nt strongly destabilize the RecA synaptic complex.[1; 2]

Controlled homologous pairing and magnetic nanomanipulation. RecA-mediated strand exchange unwinds the targeted duplex by about 1.8-fold, an effect that can be monitored *in vitro* by carrying out the reaction on a covalently closed target molecule.[3;4] Overwinding of the duplex, such as that generated by positive (+) supercoiling, stalls or inhibits complex formation.[2; 5] **Fig. 2** shows that the critical extent of intercalator-dependent (+) supercoiling required to dissociate a RecA synaptic complex on covalently closed DNA (ccDNA) is linear in the size of the homologously paired region. This implies that observing synaptic-complex dissociation as a function of externally applied torsion is a potentially sensitive technique for discriminating the size of homologous regions.

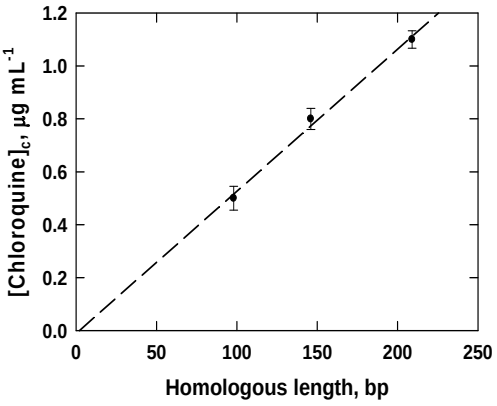

Fig. 2. Extent of positive supercoiling, in terms of critical concentration of the intercalator chloroquine, required to displace homologous RecA-DNA synaptic complexes involving human telomeric-repeat sequences. The critical concentration was determined by agarose-gel electrophoresis of RecA synaptic complexes containing ^{32}P-labeled ssDNA. Although the drug-concentration values correlate linearly with (+) supercoiling, the absolute DNA-twist change could not be determined in these experiments. The fact that the extrapolated linear dependence gives a *y*-intercept near 0 is consistent with synaptic complexes being displaced by (+) supercoiling rather than competition between intercalator and the RecA-ssDNA filament. (Zein & Levene, unpublished)

This effect can be exploited by covalently attaching 5′ and 3′ termini at one end of a dsDNA to a surface and conjugating both strands at the opposing end to a magnetic NP (see objective (**iii.**)). This approach has been used to investigate RecA-DNA interactions with single-molecule force spectroscopy[2] and lends itself naturally to a microarray-based diagnostic platform. The stability of homologous interactions between incoming RecA-coated ssDNA targets and a partially or fully homologous probe duplex will be characterized using externally applied magnetic torque. It should be noted that the small size of magnetic NPs make these nanostructures particularly suited to microarray-based applications in which DNA duplexes are closely spaced. Micron-sized iron-oxide particles, such as those used in single-molecule magnetic-trap experiments, are too large for this application (they are comparable to the size of a single microarray spot and also about an order of magnitude larger than the contour length of a dsDNA molecule in the size range of interest).

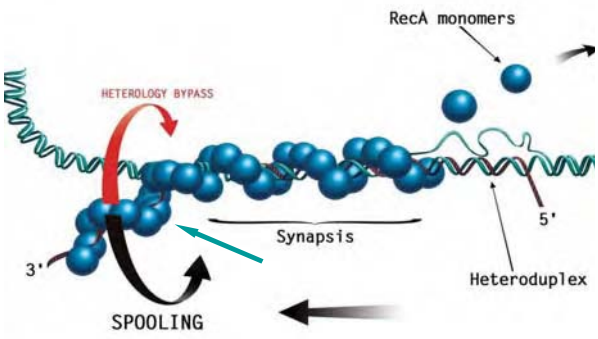

Fig. 1. In the presence of ATP, RecA (blue spheres) polymerizes on single-stranded DNA to form a presynaptic filament. During synapsis, the presynaptic filament (green arrow) is aligned with a complementary sequence in homologous duplex DNA. In linear DNAs, the synaptic complex is extended over the length of homology, followed by strand exchange, which displaces the DNA strand that is not complementary to the incoming single strand. Figure adapted from Fulconis *et al.* [2]

Task ii.

Analysis of RecA-DNA complex formation using surface-plasmon spectroscopy. Surface phenomena taking

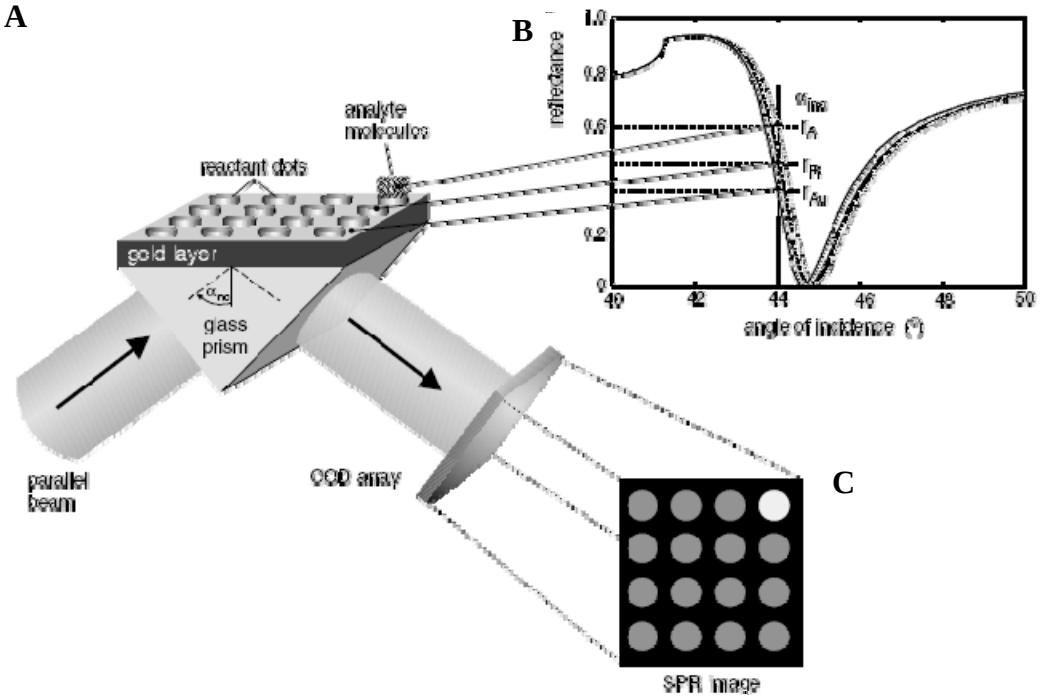

Fig. 3. Principle of surface-plasmon-resonance imaging (SPRI). (**A**) Plane (p-) polarized light illuminates a metal surface from below at an angle of incidence, α_{inc}, greater than or equal to that required for total internal reflection. (**B**) Scanning-angle SPR reflectance curves for different samples showing the increase in angle of maximum reflectance as a function of binding events occurring on the surface. Note that there is a shift in the reflectance curve observed for immobilized molecules relative to the gold substrate; an additional shift occurs on binding of analyte molecules. This is shown in (**C**) as a pattern of spots with varying intensities on the black background. The area of greatest intensity corresponds to that where analyte is bound. From Steiner [7]

place on a DNA microarray are ideally suited to monitoring by surface-plasmon-resonance imaging (SPRI).[6] In SPR, the change in refractive index of a solution at the liquid-surface interface can be determined from the change in the critical angle of refraction (**Fig. 3**).[7; 8] This technique is one of the most versatile methods currently available for high-throughput analysis of interactions among biomolecules without the use of fluorophores or other labels.

We have designed and begun construction of a high-performance SPRI instrument that incorporates an indwelling permanent magnetic-dipole field. The goal is to obtain direct mechanical readout of the sizes of RecA-dependent homologous regions by using magnetic-twist-dependent SPRI on surface-immobilized/magnetic-NP-conjugated duplex DNAs. This special application of SPRI dictates a number of important details regarding instrument and experimental design.

SPRI instrument design and construction. The principle of SPRI is based on the interaction of electromagnetic radiation (surface plasmons) propagating along a free-electron-metal surface (usually Au) with a dielectric layer in contact with the surface (**Fig. 3**). Surface plasmons are excited by p-polarized light impinging on a surface at an angle equal to or greater than the critical angle required for total internal reflection. Normally, the incident beam is coupled via a prism into a metal layer (thickness ≈ 50 nm) deposited on glass or fused-silica slides. The angle corresponding to the maximum intensity of reflected light is a sensitive function of the refractive index at the metal-dielectric interface; this refractive index is perturbed by the binding of analyte molecules to the metal surface. A shift in the angle corresponding to maximum reflectivity can be measured by rotating the detector; more typically, the change in reflectivity for a fixed angle is determined. Sensitivity of the technique is such that refractive index changes on the order of 0.01% can be detected.[7] With proper optics and control of other variables, spatial resolution in the detected image on the order of 2 μm can be achieved.[9]

In order to provide optimal sensitivity and instrument flexibility, there were several design considerations. First, a stable and compact instrument with a wide angular range was sought. The design was envisaged as encompassing capabilities for both SPRI and fluorescence imaging (possibly with changes of the light source). In both cases, imaging of the surface occurs at high angles and this dictated specific details of the detection optics. Second, the SPRI instrument had to accommodate a large magnetic dipole with a permanent field greater than 0.5 T. This is the field strength needed for magnetic manipulation of surface-immobilized RecA-DNA complexes conjugated to magnetic nanoparticles, as described in task (**iii.**). Because this field is close to

the limit of what can be achieved with even the strongest permanent magnetic materials, the design needed to take into account the presence of a 35-lb rotatable magnet assembly. The requirement for a massive magnetic dipole was incompatible with any currently available commercial instrument. Other solutions such as the use of an electromagnet were not adopted due to the difficulties involved in dissipating excess heat. Finally, we aimed to optimize overall stability of instrument response by using a stabilized HeNe light source and attending to details of mechanical vibration isolation.

A published SPRI-instrument design by Lyon *et al*.[10] provided a useful starting point. However, this published design is neither compact, nor does it provide any possibility of above-plane fluorescence detection. Moreover, the relative positions of optical elements must be readjusted for large changes in the incident and reflectance angles in order to maintain focus. To avoid these problems, we designed our instrument around a pair of coaxial rotation stages mounted on the sides of a custom-built aluminum mounting platform (**Fig. 4**). All optical components are mounted on rigid cage assemblies that can rotate over a wide range on a pair of

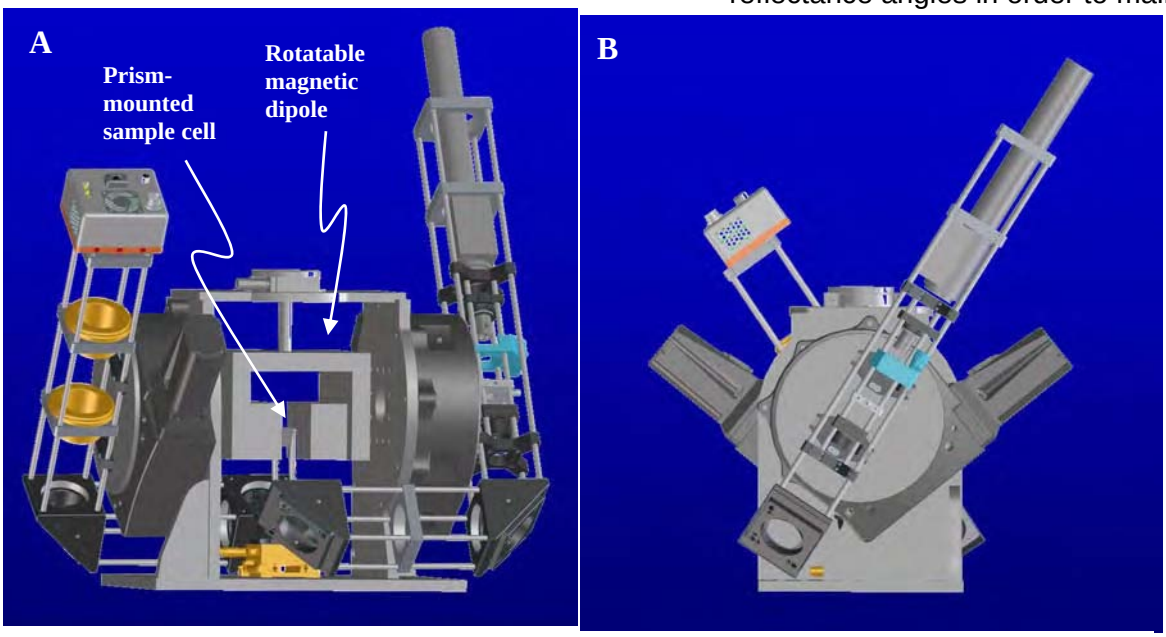

Fig. 4. CAD model of the SPRI instrument under construction. (**A**) Near-frontal view showing the illumination arm on the right and image-detection arm on the left. Layouts of optical components are specified in greater detail below. Location of the prism/sample cell assembly is indicated and the permanent-magnet dipole assembly is shown. (**B**) Side view of the apparatus, which illustrates the design's wide range of angular motion.

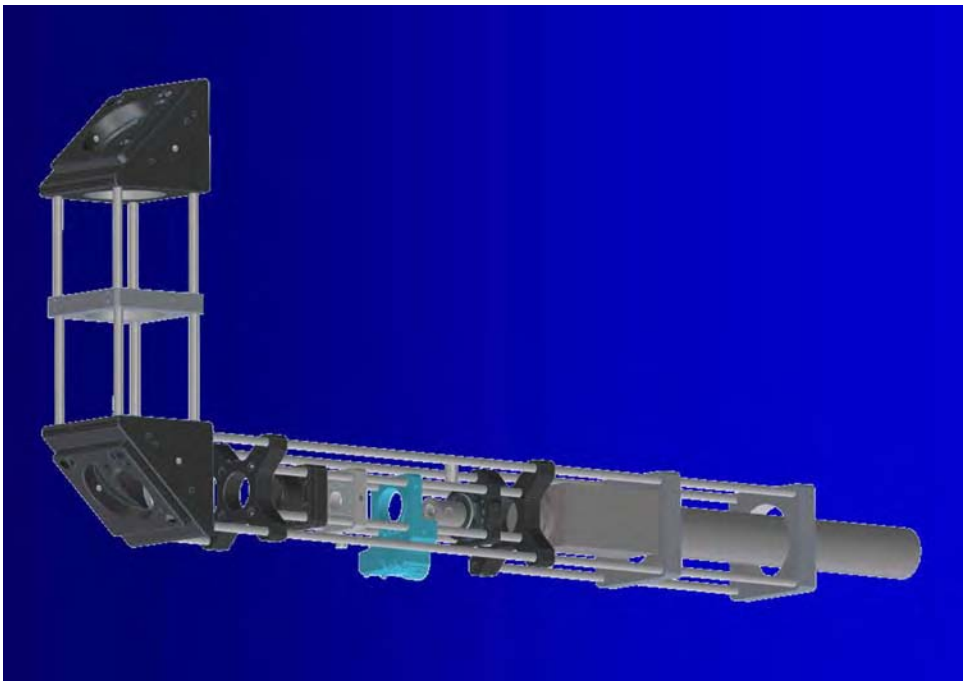

Fig. 5. CAD model of the SPRI instrument illumination arm. Components

are labeled as follows: (**a**) HeNe laser; (**b**) Glan-Thompson polarizer (**c**) aspheric lens assembly on z-axis translation stage; (**d**) pinhole on 2-axis translation stage; (**e**) 1-in collimating lens; (**f**) 2-in beam-steering mirror assembly; (**g**) 2-in, f = 350 mm lens.

Micos PRS-200 motorized open-loop-stepper rotation stages. **Fig. 5** shows a detailed view of the illumination arm of the apparatus. The incident beam is generated by a 1.2-mW, 633-nm stabilized HeNe laser (Melles Griot). The beam passes through a Glan-Thompson polarizer, spatial filter assembly (aspheric lens/20-μm pinhole/1-in collimating lens), and a 2-in, f=350 mm plano-convex lens. This optical configuration is designed to provide stable and highly uniform illumination of the sample with extremely low wavefront distortion. The folded beam path is critical to achieving uniform illumination because the 0.5-mm-diameter HeNe beam can easily be expanded to a spot size that is more than 100-fold larger at the position of the sample.

An Au-coated cover slip is pressure fitted to a custom-built flow cell that will be temperature regulated by a homebuilt Peltier device. This sample cell is optically mounted with index-matching fluid to a 70°-40°-70° prism (Tower Optics) secured by stainless-steel rods to a 3-axis translation assembly. The image-collection arm of the instrument is shown in **Fig. 6**. Because imaging of the sample surface occurs at high angles, the image is compressed in the plane of reflection. This is corrected by using a pair of hemicylindrical lenses.

The refocused beam is then collected by a high-performance EMCCD camera (Andor DL604, 1004x1002 8-μm pixels). Image acquisition, rotation-stage control, and data processing are being programmed in LabView (National Instruments), taking advantage of the availability of software-development kits sourced from component vendors. Mechanical and optical stability of the imaging system is further improved by mounting the entire apparatus on a vibration-isolated workstation (Kinetic Systems MK26).

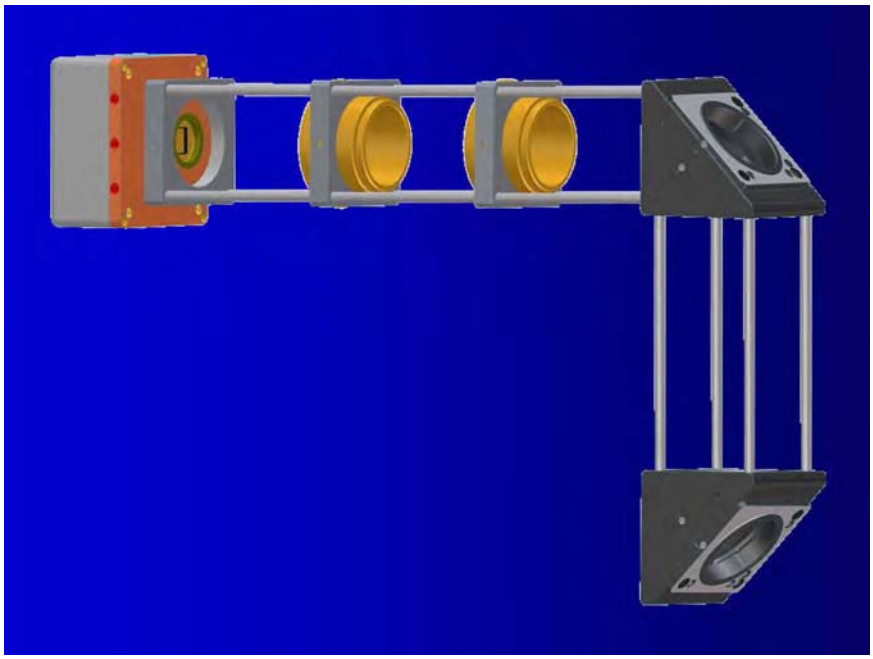

Fig. 6. CAD model of the SPRI instrument image-collection arm. Components are labeled as follows: (**a**) 2-in beam-steering mirror assembly; (**b**) 2-in f = 50 mm plano-convex hemicylindrical lens; (**c**) 2-in f = 150 mm plano-convex hemicylindrical lens; (**d**) Andor 1002x1004-pixel EMCCD camera.

Magnetic nanoparticles for biological applications. We are working with J.P. Liu's group at UT-Arlington to produce uniform, near-monodisperse FePt-based ferromagnetic NPs with diameters in the sub-10-nm size range. This size range is three orders of magnitude smaller than conventional Fe-oxide ferromagnetic beads and well-suited to applications such as macromolecular arrays. Nanoparticles with dimensions as small as 4-nm have been synthesized and have greatly enhanced magnetization (magnetic dipole moment per unit volume, 6 - $8 \cdot 10^5$ A·m^{-1}), and high room-temperature coercivity ($8 - 16 \cdot 10^5$ A·m^{-1}).[11] Particle size, morphology, and magnetic properties are well controlled and tuned in accordance with applications. Simple calculations show that rotation of a 0.5-T magnet will produce sufficient magnetic coupling to generate high levels of supercoiling in short tethered DNA fragments.

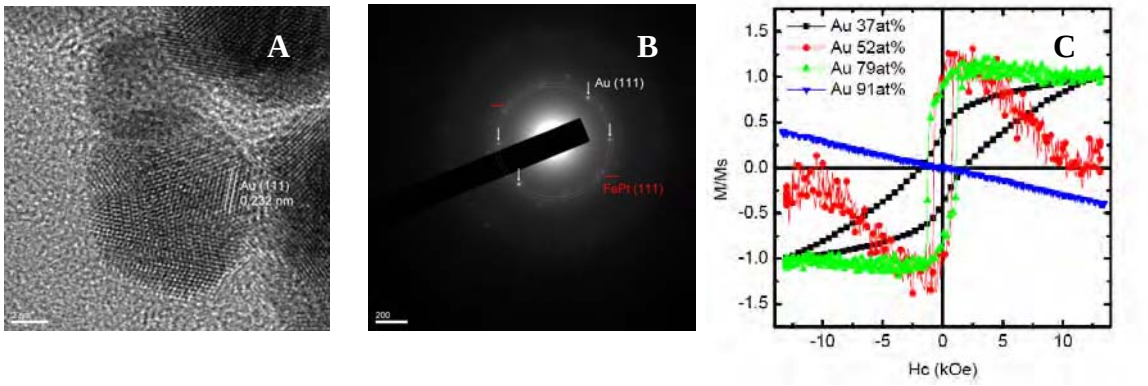

Fig. 7. Synthesis and physical properties of Au-coated FePt nanoparticles. (**A**) High-resolution transmission EM images of 6-nm FePt NPs coated with a 2- to 4-nm Au shell. The fcc lattice structure of Au can be seen with a characteristic lattice constant of 0.236 nm. Bar = 2 nm. (**B**) Nano-beam diffraction data for FePt@Au NPs, with ring diameters characteristic of Au(111) and FePt(111). (**C**) Magnetic coercitvity curves for Au-coated FePt nanoparticles. The presence of hysteresis loops, a signature of ferromagnetic behavior, can be seen for NPs containing Au up to a mole fraction of 0.79. Additional increases in Au mole fraction abolish ferromagnetic behavior.

The present challenge is to develop biocompatible coatings that support interfacial chemistry appropriate for covalent attachment to DNA yet do not significantly perturb the particles' magnetic properties. The Liu group has made substantial recent progress in developing 8-nm diameter Au-coated FePt NPs (**Fig. 7**), for covalent attachment to 5′/3′ thiol-terminated dsDNA fragments. Other coupling chemistries are also being explored such as amino-terminated DNA to silica-coated particles. There is ongoing optimization of synthesis techniques to further increase the coercivity of Au-coated NPs.

Methodology for attachment of specific DNA fragments to magnetic nanoparticles and surfaces. As in many biosensor applications, the mechanisms for linking recognition molecules to surfaces in a controlled and specific manner, and the use of non-fouling surfaces, are critical to the success of the method. We found that attaining reproducible behavior with this nanoparticle system demanded a more thorough characterization of FePt@Au nanoparticle physical properties; specifically, knowing the concentration of NPs from optical absorption measurements. The complex core-shell structure of these particles and the absence of information about the optical properties of the nanocrystalline FePt core necessitated that the NP extinction coefficent be determined empirically.

We first had to find a suitable solvent system for dispersing FePt@Au NPs and identified cyclohexanone as nearly optimal for these experiments. Visible absorption spectra could be reliably obtained in this solvent. We also found that dilute suspensions of NPs in cyclohexanone could also be deposited on highly-oriented pyrolytic graphite (HOPG) for imaging by AFM. Examples of NP images obtained on HOPG substrates are shown in **Fig. 8**. An estimate of the NP molar-extinction coefficient was therefore obtained by finding the density of particles on the surface and comparing this value with the optical absorption at 510 nm. These studies gave an extinction-coefficient value equal to $4.0 \cdot 10^9$ M^{-1} cm^{-1}.

Another outcome of these studies was a more complete picture of nanoparticle shape. The height distribution of nanoparticles in these AFM experiments appears bimodal (**Fig. 8B**), consistent with an axial ratio near 2:1. Thus, these nanoparticles are more closely resemble oblate ellipsoids than spherically symmetric clusters.

Key Research Accomplishments

- Development and construction of a novel surface-plasmon-imaging instrument capable of analyzing magnetically-controlled DNA hybridization experiments in a high-throughput format

- Synthesis and characterization of Au-coated FePt ferromagnetic nanoparticles suitable for conjugation to DNA and other biomolecules

Reportable Outcomes

Manuscripts:

Shoura, M.J., Vikas, N., Kazuaki, Y., Liu, J.P., and Levene, S.D. Development and properties of ferromagnetic nanoparticle-DNA conjugates. *Manuscript in preparation.*

Grant applications:

Advanced DNA Microarray Technologies Based on Magnetic Nanomanipulation (PI: Levene, co-PIs: Liu, Hu, Hanke). Texas Advanced Research Program, $150,000 (T/DC)

Interfacial Chemistry for Magnetic Nanomanipulation of DNA and Protein-DNA Complexes (PI: Levene, CINT collaborators: Martinez, Dattelbaum, Huber). LANL/CINT User Program.

Conclusions

We have devised a method for real-time, high-throughput monitoring of macromolecular interactions under conditions where magnetic nanomanipulation of target molecules can be achieved. The proposed application of this technology is a new microarray-based platform for fine-structure mapping of genomic rearrangements based on RecA-mediated strand exchange. This technology is a potentially valuable complement to cytogenetic and other hybridization-based methods for the clinical diagnosis and staging of cancer and other diseases involving DNA rearrangements. A key element of this program is the advancement of an effective imaging method for interrogating a microarray surface subjected to a strong magnetic field. No commercial instrumentation is available for this application; thus, we have designed and constructed a high-performance surface-plasmon-resonance-imaging (SPRI) system that addresses this need. In fact, the instrumentation has capabilities that go beyond SPRI and can be operated in an evanescent-illumination, epi-fluorescence mode.

In addition, we have carried out a more complete characterization of Au-coated ferromagnetic nanoparticles, which are an integral component of the magnetic- nanomanipulation approach. This work has resulted in improved protocols for nanoparticle handling and quantitation, which are essential to controlling interfacial chemistry for DNA coupling and also minimizing undesirable interactions between the nanoparticle and biological macromolecule.

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of variables and a simple but powerful mathematical construct to integrate them. 4) Related to GSPT is the Elemental Resource Model of the human, which provides a way to break down HP resources into meaningful, measurable entities. Combined, these models may allow optimization to be performed with limited knowledge of the factors to be integrated into the whole. 5) Finally, Human Performance Operator Optimization (HPO2) is a team approach at the operational level, centered on the customer. HPO2 is predictive, and derives its power from integrating the perspectives of its carefully chosen team members. **Discussion:** Comparing and contrasting these approaches provides a rich context for considering HP and how to optimize HP, both within a narrow context such as a specific task or mission, and in a broader sense, such as an occupation or career.

Learning Objectives: 1. Mathematical and graphical methods can be applied to optimization of human performance. 2. Human performance optimization is an integrative function that takes place at the operational level.

[182] ARCHETYPE CONSTRUCTS AS A BASIS FOR HUMAN PERFORMANCE OPTIMIZATION

A. RUSSELL and B. BULKLEY
Scitor Corporation, Rosslyn, VA

Defining and measuring different kinds of human performance is a nearly universal challenge for teams, organizations, and industries. Biotechnological innovations show tremendous potential for modifying human physical, cognitive, and affective attributes, but current task-oriented analyses are poor tools to understand how these biotechnologies could affect military operator performance. We developed an alternate method for analyzing, representing, comparing, and potentially optimizing military human performances of different kinds. Key human performance attributes were identified that span the dimensions of physiology, cognition, and emotion. Participant-observation research was then conducted into human performance demands for different military units and personnel, and the Human Performance Archetype concept was developed from the findings. The Archetype graphically places different human attributes in relationship to each other and to the performance demands made on personnel by a unit's mission, its culture, and its operational environment. The Archetype also allows us to compare human performance differences across units, which would otherwise be unfeasible if using tasks to compare units. By graphically representing human performance as a simultaneously physical, cognitive, and socioemotional phenomenon, the Archetype suggests a more comprehensive battery of human performance metrics to be developed in order to optimize that performance, whether through personnel selection, training, or even the use of performance biotechnologies. Finally, the Archetype can also help clarify the notion of "Human Performance Capital", which refers to the physical, cognitive, and socioemotional resources that a person possesses to answer specific performance demands. Our presentation will briefly cover the development of the Archetypes, explore both the advantages and disadvantages an Archetype-like approach to human performance presents, and discuss the value of a more general notion of Human Performance Capital for future research into military HPO.

Learning Objectives: 1. To present alternative methods for analyzing military human performance needs.

[183] CONCEPTUAL VISION FOR AN AIR FORCE HUMAN PERFORMANCE DOCTRINE: PERFORMANCE OPTIMIZATION

L. BROWN[1] and A. P. TVARYANAS[2]
[1]311th Human Systems Wing, San Antonio, TX; [2]Naval Postgraduate School, Salinas, CA

Introduction: Human Performance (HP) is one of the core competencies which Health Service Support (HSS) personnel provide as a force enabler for both in-garrison and deployed military operations. Advocating for HP is challenging because of the immaturity of HP doctrine relative to other HSS competencies (e.g., preventive medicine, casualty management, etc.). Recent work has attempted to provide a conceptual blueprint for an overarching HP doctrine, and this paper presents the section specifically dealing with Human Performance Optimization (HPO). **Methods:** A panel of U.S. Air Force HP subject matter experts held a 2-day workshop in July 2007 at Langley AFB to develop a draft HP doctrine. Subsequently, inputs on the draft proposal were solicited from HSS command leadership. **Results & Discussion:** HPO was defined as those activities which seek to achieve the most efficient use of limited human resources by comprehensively integrating humans within larger socio-technical systems and Human Systems Integration (HSI) was identified as the supporting process model. Additionally, a modified version of the traditional HSI model was adopted based on recent theoretical work by the Naval Postgraduate School. In this model, four domains (human factors engineering, personnel, training and manpower) are input domains, whereas the other three domains (Environment, Safety and Occupational Health (ESOH); habitability and survivability) are first order effects and HP is a second order effect of the input domains. Importantly, the latter three domains describe the HSS function of Force Health Protection (FHP), allowing FHP to be addressed through the systems engineering process. The proposed model also offers the potential for using data from FHP failures (i.e., HFACS databases) to define individualized HSI models for systems which could be the foundation for quantitative HSI cost-benefit analyses.

Learning Objectives: 1. Doctrinal solutions to human performance optimization will be discussed. 2. The audience will learn how the Human Systems Integration model can be used to optimize human performance.

[184] GENERAL SYSTEMS PERFORMANCE THEORY AND THE ELEMENTAL RESOURCE MODEL FOR HUMAN PERFORMANCE

G. V. KONDRASKE
Univ. of Texas at Arlington, Arlington, TX

Introduction: General Systems Performance Theory (GSPT), although applicable to any systems-task, was motivated by challenges in human performance. To gain insight into fundamental principles, human system complexity was set aside and focus was placed on simple hypothetical systems and tasks. GSPT thus provides a first principles, conceptual, quantitative, and hierarchical framework for modeling systems, tasks, and their interface using abstraction of these items that focuses on the notion of "performance". **Methods & Results:** With GSPT, systems (and/or subsystems) are modeled as possessing a set of "performance resources" that reflect the unique qualities that characterize "how well" a given system executes its function (e.g., accuracy, speed, strength, endurance, etc.). The nonlinear, threshold-oriented mathematics of resource economics are incorporated. In this model, Resource Availability must exceed Resource Demand for "success" of a given system in a given task. Tasks draw upon multiple system performance resources; this thus extends to the logical combination of resources, which may be written as "sufficiency" (an amount ≥ threshold) is required for Resource A AND B AND C, etc. The concept of a performance capacity envelope is derived from this representation, the volume of which represents the capacity of the system to perform tasks that make demands on performance resources that form the multi-dimensional performance space. This suggests a multiplicative computation to characterize a generic capacity to perform. Applying these concepts and that of monadology to the human system results in the Elemental Resource Model. **Discussion:** The redundancy present in the human system and the constructs of GSPT suggest a new approach to the problem of optimization; i.e., the system (human) is driven to accomplish the goal (task) in a manner that minimizes the stress on all performance resources involved in task execution (i.e., maximizing the margin between available resources and utilized resources).

Learning Objectives: 1. To understand the basic constructs of General Systems Performance Theory and application to human performance modeling and measurement. 2. How performance resources are defined and used to modeling any aspect of human and human subsystem performance. 3. Learn the structure of the Elemental Resource Model for human-task interfaces.

[185] GENERAL SYSTEMS PERFORMANCE THEORY AND HUMAN PERFORMANCE: SOME EXPERIMENTAL RESULTS

University of Texas at Arlington, Arlington, TX

Introduction: Concepts of General Systems Performance Theory (GSPT) and monadology were applied to the human system to realize the Elemental Resource Model (ERM). A task analysis, performance modeling and performance prediction methodology dubbed Nonlinear Causal Resource Analysis (NCRA) was also developed using GSPT. NCRA not only estimates the level of performance in a higher level task (HLT) supported by a set of lower level or basic performance resources (BPRs), but also identifies which BPRs limit HLT performance. **Methods & Results:** Overviews in applications where the HLT is a complex sport task, driving, and laparoscopic surgery are presented. In each context, an ERM-guided gross task analysis is first performed to identify a set of BPRs (i.e., specific performance capacities for specific human subsystems) involved in HLT execution. This initial set is prioritized and reduced, taking into account time allowable for performance capacity measurements. To build performance models, data is collected at the BPR and HLT levels. BPR

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capacities are measured using a variety of objective maximal performance techniques that produce single number results reflecting "how much" of a given BPR is available. HLT performance is measured subjectively by one of more domain experts that observe subjects and rate integrated performance on a visual-analog scale. Custom NCRA software produces Resource Demand Functions (estimates of the amount each performance resource utilized to achieve a given level of HLT performance), uses these functions to predict HLT performance, and identifies limiting performance resources. Given the relatively small number of BPRs included in each model, experimental results have been encouraging; differences between predicted and expert-rated performance ≤ 15% for a large fraction (e.g. 75-90%) of the study populations. **Discussion:** Where differences are large and predicted exceeds expert-rated performance, it is likely that the subject is limited by a BPR that was not included in the model.

Learning Objectives: 1. To learn about the logic behind Nonlinear Causal Resource Analysis. 2. To learn how to apply Nonlinear Causal Resource Analysis and the Elemental Resource Model to develop performance models for specific contexts.

[186] HUMAN PERFORMANCE OPTIMIZATION AT THE BASE LEVEL

P. NELSON
14th Medical Group, Columbus Air Force Base, Mississippi, Columbus AFB, MS

The Human Performance Operator Optimization (HPO2) Team is a base level cross functional team of Line and Medical assets, answering the operator's need for performance enhancement expertise for our next generation warfighter. The team solicits and prioritizes opportunities to enhance both individual and system performance, and recommends action through ad hoc Tiger Team formation. The HPO2 team supports these individual Tiger Teams with performance enhancement expertise, and forwards a vetted capability gap analysis through leadership to the MAJCOM for broader consideration. Discussing the HPO2 organizational structure and information flow provides an opportunity to identify possible solutions to base level integration of line and medical human performance assets with MAJCOM and Air Force human performance assets. The views presented are those of Dr Nelson and do not reflect the views of the United States Air Force.

Learning Objectives: 1. Understand challenges and possible solutions to local implementation of integrated human performance teams.

Tuesday, May 13 *8:30AM*

SLIDE: Human Error in Aviation

[187] COGNITIVE PROCESSES ASSOCIATED WITH THE LOSS OF SITUATION AWARENESS

L. L. BAILEY, J. POUNDS and A. L. SCARBOROUGH
FAA-CAMI, Oklahoma City, OK

Purpose: The loss of situational awareness (SA) is the most common human error identified by en route air traffic control (ATC) quality assurance (QA) personnel charged with investigating ATC operational errors (OEs). The SA construct involves integrating several cognitive processes, and therefore, it is not always clear what is meant when an ATC OE investigator identifies a loss of SA as an OE causal factor. We attempt to elucidate the operational meaning of a loss of SA by using an OE investigation tool called JANUS-ATC. **Method:** Two OE investigation techniques were simultaneously conducted by separate OE investigators for 67 en route OEs that occurred during 2002. The first technique used the current OE investigation process that assesses three components of SA: (a) detection, (b) comprehension, and (c) projection of future status. The second technique used a human factors diagnostic tool called JANUS-ATC, which classified human errors as: (a) perception and vigilance, (b) memory, and (c) planning and decision making. The results of the two techniques were used to construct a 3x3 contingency table to determine the degree to which a given SA component was associated with a given JANUS-ATC category. **Results:** Planning and decision making was the most common JANUS-ATC category associated with each of the three respective SA components (63%, 63%, & 65%). Next was perception and vigilance (32%, 31%, & 20%) and last was memory (26%, 13%, & 16%). **Discussion:** The results suggest that when ATC OE investigators attribute an OE to a lack of SA, they are implying that the controller more than likely demonstrated poor planning and decision making. However, it remains unclear whether the lack of planning and decision making was associated with poor tactical or poor strategic decisions. Thus it appears that a temporal dimension of SA is needed to better capture what is meant by a lack of SA.

Learning Objectives: 1. to learn a new air traffic control taxonomy of human error. 2. to understand how to customize training based on cognitive processes.

[188] LOGISTIC REGRESSION ANALYSIS OF OPERATIONAL ERRORS AND ROUTINE OPERATIONS IN EN ROUTE AIR TRAFFIC CONTROL

E. PFLEIDERER and C. SCROGGINS
FAA CAMI, Oklahoma City, OK

Purpose: Numerous studies have examined environmental elements contributing to operational error (OE) occurrence. Most have been done without reference to routine operations (ROs). Yet, for every OE that occurs in a sector, there are hundreds (possibly thousands) of hours in which an OE did not occur. To truly understand the contextual factors that contribute to OEs, it is necessary to identify what was different about the sector environment at the time. The Number of Aircraft with Lateral Distances <10 nm, Transitioning Aircraft, Heading Changes, Handoffs, Point Outs, Average Vertical Distance, and Average Control Duration were submitted as predictors in a backward stepwise logistic regression analysis to determine how well dynamic traffic characteristics could discriminate between OE and RO traffic samples. **Method:** OE traffic samples were derived from SATORI (Systematic Air Traffic Operations Research Initiative; Rodgers & Duke, 1993) re-creations of OEs occurring at the Indianapolis Air Route Traffic Control Center (ARTCC) between 1/5/2003 and 12/10/2003. Sector characteristic variables were computed in 5-minute intervals for each OE (i.e., 4 minutes prior, 1 minute after) and summarized (averaged) for each sector. RO traffic samples were extracted from System Analysis Recordings (SARs) taped between 5/8/2003 and 5/10/2003, and sector characteristics variables were computed in 5-minute intervals and summarized (averaged) for each sector. This produced a total of 65 observations (28 OEs and 37 ROs). **Results:** Variables included in the final model (Number of Aircraft with Lateral Distance <10 nm, Number of Transitioning Aircraft, Number of Handoffs, and Average Control Duration) accurately classified OE and RO samples for 91% of the traffic samples. **Conclusions.** Although the results of the logistic regression analyses cannot be used to determine causation, they effectively identified variables that distinguished between OE and RO traffic samples. Further research is required to test how well these results generalize to other facilities.

Learning Objectives: 1. The relationship between sector characteristics and the occurrence of operational errors in en route airspace is described.

[189] A LONGITUDINAL EXAMINATION OF THE RELATIONSHIP BETWEEN OPERATIONAL ERRORS AND TRAFFIC VOLUME

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Introduction: Human factors analyses have established strong and fairly consistent positive relationships between the number of aircraft operations and the number of Air Route Traffic Control Center (ARTCC) operational errors (OEs). Given that aircraft operations are expected to increase by 33% by the year 2015, there should be a corresponding increase in the number of OEs. However, it remains unclear if the expected increase would occur across all ARTCCs or within a restricted few. To help answer this question, we conducted a longitudinal study of OEs at the ARTCCs. **Method:** Number of aircraft operations and OEs were compared across 21 ARTCCs from the year 1995 though 2006. Scatter plots and regression lines for each year were examined. R2 values were compared across years. **Results:** R2 values across all ARTCCs ranged from .44 in 1996 to .81 in 2003, which produced an average R2 of .61. However, six ARTCCs consistently appeared as outliers on the scatter plots. This suggested the existence of two populations, those above (n=6) and those below (n=15) 2,100,000 annual aircraft operations. The average R2 for the higher operations ARTCCs was .19 and the average R2 for the lower operations ARTCCs was .47. **Discussion:** If the increase in operations is uniform across centers, some ARTCCs will likely experience a corresponding increase in OEs and others will not. We can expect to see an increase in OEs at the lower operations ARTCCs as the number of operations increase. However, since the higher operations ARTCCs fall into a different population, it is uncertain how an increase in aircraft operations would affect their OEs. Perhaps for the latter, sector characteristics (i.e.

Thermal ablation of tumor cells with antibody-functionalized single-walled carbon nanotubes

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Single-walled carbon nanotubes (CNTs) emit heat when they absorb energy from near-infrared (NIR) light. Tissue is relatively transparent to NIR, which suggests that targeting CNTs to tumor cells, followed by noninvasive exposure to NIR light, will ablate tumors within the range of NIR. In this study, we demonstrate the specific binding of antibody-coupled CNTs to tumor cells *in vitro*, followed by their highly specific ablation with NIR light. Biotinylated polar lipids were used to prepare stable, biocompatible, noncytotoxic CNT dispersions that were then attached to one of two different neutralite avidin-derivatized mAbs directed against either human CD22 or CD25. $CD22^+CD25^-$ Daudi cells bound only CNTs coupled to the anti-CD22 mAb; $CD22^-CD25^+$ activated peripheral blood mononuclear cells bound only to the CNTs coupled to the anti-CD25 mAb. Most importantly, only the specifically targeted cells were killed after exposure to NIR light.

immunoconjugates | lymphoma cells | monoclonal antibodies | nanotechnology | near infrared light

Despite the success of current treatments for several types of cancer, all known treatments have major limitations. Conventional chemotherapy or radiotherapy damage many cells, and both have significant side effects. In addition, tumor cells develop resistance to many chemotherapeutic agents (1), and most chemotherapeutic drugs kill dividing cancer cells and not dormant ones. To decrease nonspecific toxic effects and kill nondividing cells, targeted therapies are being developed and some have already been approved by the Food and Drug Administration for use in humans. These include both small molecules that target specific intracellular pathways in tumor cells and mAbs that target molecules on their surface. Some of these targeted agents are cytostatic and not cytotoxic, and they are often given in combination with chemotherapy in an effort to both lower the dose of chemotherapy required and hence reduce side effects and achieve additive or synergistic effects. With regard to mAbs, strategies include increasing cytotoxicity by coupling them to drugs, radionuclides, toxins, drugs, or prodrugs (2, 3). These agents (collectively called immunoconjugates) are potent, and three have been approved for human use (4, 5). However, they also have side effects because they carry toxic payloads. We and others have successfully tested the antitumor activity of different agents, including signaling antibodies and immunotoxins, alone or in combination with pharmacological agents, in disseminated or solid human tumors grown in immunocompromised mice (5–9). We have also tested four different immunotoxins in humans (10–15). To optimize the use of mAbs in cancer therapy, it is important to explore their use with new types of payloads and carriers, including carbon nanotubes (CNTs). The ability of CNTs to convert near-infrared (NIR) light into heat provides an opportunity to create a new generation of immunoconjugates for cancer photo-therapy with high performance and efficacy. Moreover, hyperthermia has been clinically used in the management of solid tumors because it can synergistically enhance tumor cytotoxicity when combined with chemotherapy or radiotherapy (16, 17). Hyperthermia also preferentially increases the permeability of tumor vasculature compared with normal vasculature, which can enhance the delivery of drugs into tumors. Therefore, the thermal effects generated by targeted CNTs may have important advantages. Recent pharmacokinetic studies have reported that CNTs dispersed by different procedures lack nonspecific toxic effects in mice (18–20).

The use of NIR-resonant nanostructures, including gold nanoshells and CNTs, to thermally ablate cancer cells is being explored by several groups (21–26). The use of NIR light in the 700- to 1,100-nm range for the induction of hyperthermia is particularly attractive because living tissues do not strongly absorb in this range (27). Hence, an external NIR light source should effectively and safely penetrate normal tissue and ablate any cells to which the CNTs are attached. The critical aspect for selective CNT-mediated thermal ablation of cells is to stably attach targeting moieties that will not interfere with the optical properties of the CNTs and yet retain targeting specificity. The targeting of CNTs to tumor cells can be accomplished by coating them with cell-binding ligands such as peptides or mAbs (25, 26, 28–30). Several studies have reported that the targeting of such CNTs is "specific" (25, 26, 29, 30), but no study has used both a control ligand and a control cell to convincingly demonstrate ligand-specific thermal ablation of tumors cells with CNTs. Specificity is critical because nonspecific binding to antigen-negative cells *in vivo* could cause major side effects, which has been a confounding issue in the cancer targeting field for >25 years.

The aim of this study was to design and prepare an anti-CD22-targeted CNT construct to ablate human Burkitt's lymphoma cells *in vitro*. Herein, we describe the physical properties of these CNT constructs, their selective binding to tumor cells, and the NIR-induced thermal ablation of the targeted tumor cells. Importantly, both a control CNT construct and a control cell were used to definitely prove specificity.

Results

Dispersion of CNTs. Well dispersed single-walled CNTs were prepared by sonicating CNTs in the presence of 1,2-distearoyl-

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Conflict of interest statement: E.S.V., R.K.D., P.P., and I.H.M. are affiliated with Medical Nanotechnologies, Inc. E.S.V. is a coinventor on an issued patent encompassing this work.

†P.C. and R.M. contributed equally to this work.

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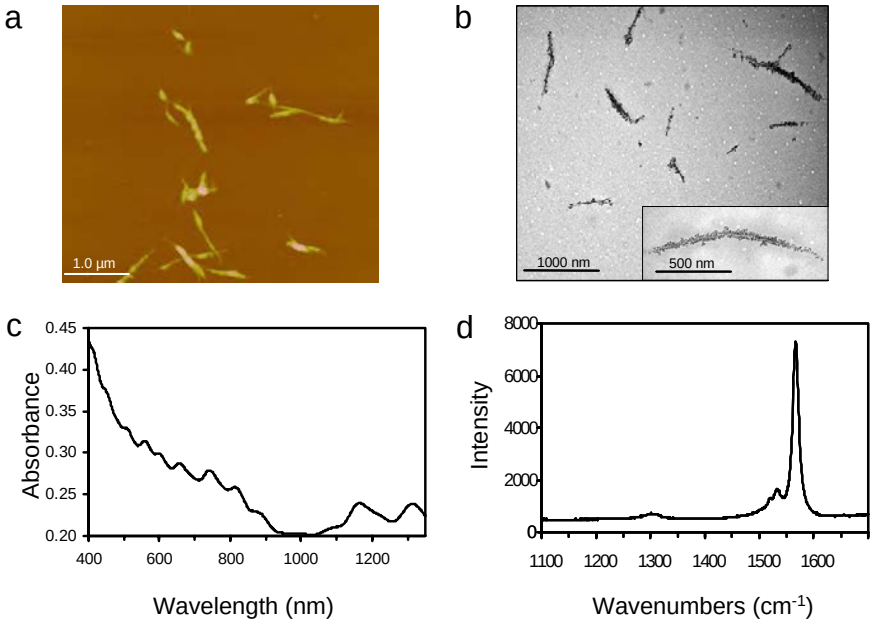

Fig. 1. Water-soluble CNTs functionalized with biotinylated polar lipids. (*a*) AFM image of B-CNTs shows CNTs coated by the biotinylated polar lipid, DSPE-PEG-biotin. (*b*) TEM images of individual B-CNTs show uniform coverage of biotin after immunodetection with gold-labeled anti-biotin. (*Inset*) Higher magnification of a B-CNT coated with gold-labeled antibiotin. (*c*) UV-Vis-NIR spectrum of B-CNTs show a number of metallic and semiconducting CNT absorbances consistent with the presence of individual tubes. (*d*) Raman spectra of B-CNTs show an intense G band (≈1,590 cm^{-1}) indicating the presence of CNTs. In all cases, one representative experiment of at least three independent experiments is shown.

sn-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol) 2000] [DSPE-PEG(2000)-biotin], followed by centrifugation to recover the biotinylated CNTs (B-CNTs). The resulting B-CNT suspension contained 0.06 mg CNT/ml and ≤3 parts per million metals, as determined by thermal gravimetric analysis (TGA) and inductively coupled plasma mass spectrometry (MS) (data not shown). The dispersions were stable and did not aggregate at room temperature for >120 days. Atomic force microscopy (AFM) analysis demonstrated that the suspension was free of nontubular carbon structures and the CNTs were either individually dispersed or in small bundles. The lengths of the CNTs ranged from 0.2 to 1.4 μm with an average of 0.59 μm (Fig. 1*a*). Analysis by transmission electron microscopy (TEM) of the B-CNT samples probed with gold-labeled goat antibiotin demonstrated that biotin was distributed along the entire surface of the B-CNT (Fig. 1*b*). The biotin content of the B-CNT dispersion was determined by using a competitive ELISA and adding dilutions of the B-CNT dispersion to biotin-HRP and plating them onto neutralite avidin (NA)-coated plates. The amount of HRP-labeled biotin was detected by the development of color in the presence of the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate. Using this assay, we found that the content of biotin was 0.02 mmol/g of B-CNT. The UV-visible (Vis)-NIR spectra of the B-CNTs confirmed the quality of these dispersions with the presence of electronic transitions between van Hove singularities, suggesting that the optical properties of the CNTs were maintained after the adsorption of DSPE-PEG-biotin (Fig. 1*c*). The Raman spectra of the B-CNTs showed a number of well characterized CNT resonances such as the radial breathing mode region between 100 and 300 cm^{-1} (data not shown) and the tangential (G-band) peak at 1,590 cm^{-1}, confirming the presence of CNTs in the sample (Fig. 1*d*).

To determine whether B-CNTs were inherently cytotoxic (in the absence of NIR), cells from the IgM^+ $CD22^+CD25^-$ Burkitt's lymphoma cell line Daudi were incubated for 24 h with up to the highest amount of B-CNTs used in the binding and killing assays (3.6 μg). No toxicity was observed using a [^{3}H]thymidine incorporation assay (data not shown).

Preparation of mAb-NA Targeting Moieties. To prepare the targeting agents, we coupled NA to mAbs. To this end, mouse IgG anti-human CD22 (RFB4) or mouse IgG anti-human CD25 (RFT5) were thiolated with 2-iminothiolane (Traut's reagent). NA was activated with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). Then, the purified thiolated IgG was mixed with MBS-activated NA at a 2:1 ratio as shown by preliminary experiments to give the best yields as determined by the elution profile of the resulting conjugate on a Sephacryl S-300 HR column (Fig. 2*a*). The concentration of the mAb-NA conjugates was determined by using the bicinchoninic acid assay (BCA). Both mAb-NA conjugates were free of impurities as judged by Western blot analysis (Fig. 2*a*). We next determined whether these conjugates were cytotoxic to Daudi cells after incubation of cells for 24 h with up to 10 μg/ml of RFB4-NA; cytotoxicity was determined by [^{3}H]thymidine incorporation. Similar concentrations of unconjugated RFB4 and NA were used as negative controls, and goat anti-IgM (which induces apoptosis of Daudi cells) (31) was used as the positive control. We found that the RFB4-NA conjugates were not cytotoxic, whereas (as predicted) the goat anti-IgM reduced [^{3}H]thymidine incorporation by >50% (Fig. 2*b*).

The specific binding of RFB4-NA and RFT5-NA conjugates to $CD22^+CD25^-$ Daudi cells and $CD22^-CD25^+$ phytohaemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs), respectively, was demonstrated by flow cytometry, using either FITC-labeled goat anti-mouse Ig (GAMIg) or FITC-biotin (data not shown for FITC-biotin). The latter was confirmed by the ability of the cell-bound mAb-NA to bind to B-CNT. Daudi cells were precoated with a saturating concentration of RFB4-NA, washed, and incubated with increasing amounts of B-CNTs. The RFB4-NA, but not the RFT5-NA conjugate could target an average of 0.237 pg of B-CNTs per cell (Fig. 2*c*).

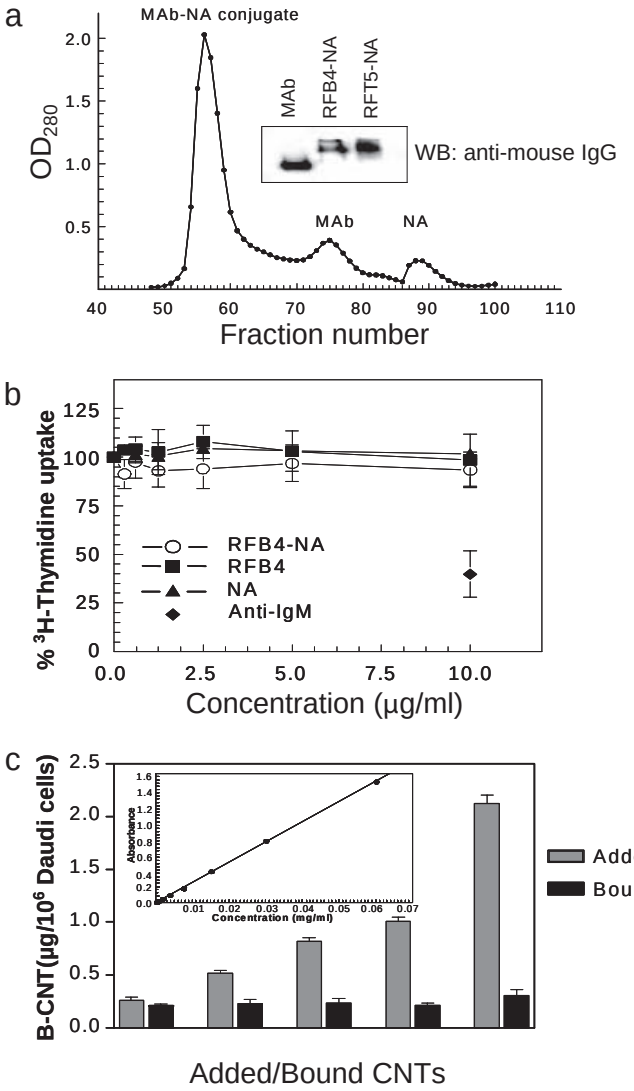

Fig. 2. Analysis of mAb-NA conjugates. (*a*) A typical chromatographic separation of RFB4-NA from unconjugated RFB4 and NA using a Sephacryl S-300 HR column. Fractions of the first peak containing the RFB4-NA conjugate were pooled and concentrated. (*Inset*) Purified RFB4-NA, RFT5-NA, or mAb were electrophoresed under nondenaturing conditions on a 7.5% polyacrylamide gel and immunoblotted with HRP-labeled sheep anti-mouse IgG. Data in *a* are representative of at least three independent experiments. (*b*) A total of 5×10^4 Daudi cells were incubated for 24 h with increasing amounts of the RFB4-NA conjugate, and cytotoxicity was detected by [^{3}H]thymidine incorporation. Similar concentrations of unconjugated RFB4 or NA were used as negative controls, whereas 10 μg/ml goat anti-IgM was used as positive control. Data represent mean ± SD of three independent experiments. (*c*) One million Daudi cells precoated with a saturating concentration of RFB4-NA were incubated with increasing amounts of B-CNT. Saturating concentration of RFB4-NA can target 0.237 pg of B-CNT per Daudi cell. No detectable B-CNT binding was found on uncoated cells or cells precoated with RFT5-NA (control). Data represent mean ± SD of three independent experiments.

Preparation and Testing of mAb-CNT Complexes. We next prepared the mAb-CNT conjugates by coupling the B-CNTs to either RFB4-NA or RFT5-NA for 35 min at room temperature. After the removal of the supernatant containing the unreacted mAb-NA, the optical properties of the freshly prepared mAb-CNT were tested. The UV-Vis-NIR spectra of the mAb-CNT conjugates displayed the same metallic and semiconducting CNT types as observed for the B-CNTs, indicating that the optical properties of the CNTs were not affected by the coupling (Fig. 3*a*), and the characteristic CNT resonances displayed in the Raman spectra of the mAb-CNTs again confirmed the presence of CNTs in the sample (Fig. 3*b*).

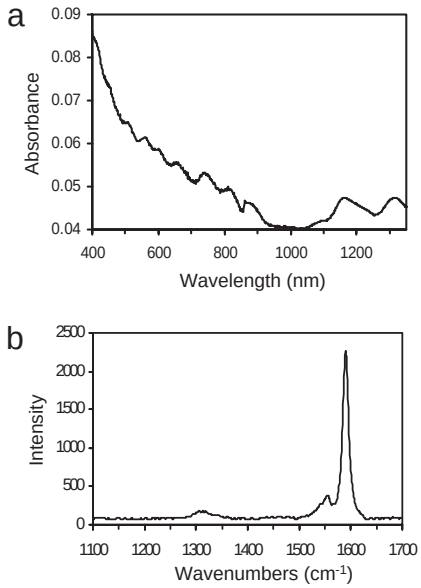

Fig. 3. Optical properties of CNTs following coupling with mAbs (mAb-CNT). (*a*) UV-Vis-NIR spectrum of RFB4-CNTs show the same metallic and semiconducting CNT types as observed for the B-CNTs, indicating the retention of the optical properties of CNTs after the coupling with RFB4-NA. The sharp feature at 861 nm is caused by a grating and detector change associated with the spectrometer. (*b*) Raman spectrum of RFB4-CNTs show an intense G band (≈1,590 cm^{-1}) as the B-CNTs, indicating the presence of CNTs in the conjugate. The spectra are representative of three independent experiments.

The ability of the mAb-CNT conjugates to bind to antigen-positive but not antigen-negative target cells was assessed by flow cytometry. The components of the cell-bound mAb-CNT were detected by using FITC-GAMIg (which binds to mouse mAb) and phycoerythrin-streptavidin (PE-SA) (which binds to biotin), respectively. We found that RFB4-CNT and RFB4 (positive control) bound equally well to Daudi cells, whereas RFT5-CNT (negative control) bound poorly ($P < 0.001$) (Fig. 4*a*). Conversely, RFT5-CNT and RFT5 bound equally well to $CD22^-CD25^+$ PHA-activated PBMCs (95% $CD25^+$ cells), whereas the negative control conjugate, RFB4-CNT, did not ($P < 0.002$) (Fig. 4*b*). These results demonstrate that the coupling of the mAbs to CNTs does not alter their mAb-binding activity and that the mAb-CNTs bind to antigen-expressing cells as specifically as the uncoupled mAbs.

Having demonstrated that the mAb-CNT conjugates retained the binding activity of the mAb and the optical properties of the CNTs, we next determined whether cells targeted by the mAb-CNTs could be thermally ablated after exposure to NIR light. Cells were incubated with the mAb-CNTs in PBS, washed three times with PBS, and then dispensed into 96-well plates in cell culture media. The cells in the plate were exposed to an 808-nm laser (5 W/cm^2) for 7 min and pulsed for the next 12 h with 1 μCi [^{3}H]thymidine to assess cell viability. As shown in Fig. 5*a*, as compared with treatment with the nonbinding RFT5-CNTs, the viability of the RFB4-CNT-treated Daudi cells was significantly reduced after exposure to NIR light ($P < 0.0001$). Conversely, when activated PBMCs were used as target cells, RFT5-CNT, but not RFB4-CNT, killed the cells after exposure to NIR light ($P < 0.0001$) (Fig. 5*b*). These experiments demonstrate that the binding of the mAb-CNTs to their respective antigen-positive target cells leads to their specific ablation after exposure to NIR light.

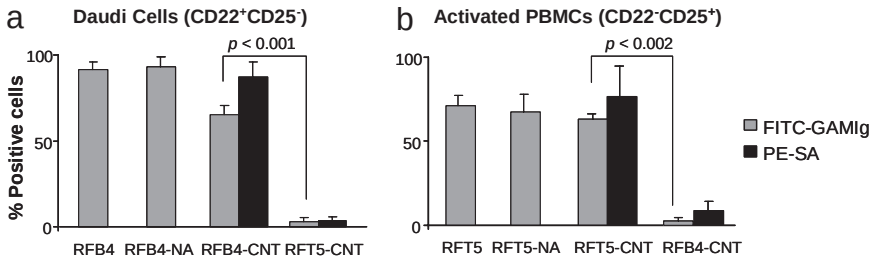

Fig. 4. Binding of mAb-CNTs to target cells. One million cells were incubated with saturating concentrations of RFB4-CNTs or RFT5-CNTs and then incubated either with FITC-GAMIg to detect the mAbs or with PE-SA to detect the B-CNTs and analyzed on a FACScan. (*a*) The specific binding of RFB4-CNTs to Daudi cells using RFT5-CNTs as a negative control ($P < 0.001$). (*b*) The specific binding of RFT5-CNT to activated PBMCs (>95% T cells) using RFB4-CNT as a negative control ($P < 0.002$). Data represent mean ± SD of at least three independent experiments.

Because we anticipate using these mAb-CNTs *in vivo*, it was important to demonstrate that they retained activity in serum at 37°C. Therefore, the mAb-CNTs were incubated in mouse serum at 37°C for 0, 24, 48, and 72 h. At each time point, the mAb-CNTs were washed with PBS, incubated with Daudi cells, and irradiated with NIR light in a procedure similar to the thermal ablation described above. No loss in their ability to thermally ablate Daudi cells was observed, even after 72 h in mouse serum at 37°C (Fig. 5*c*).

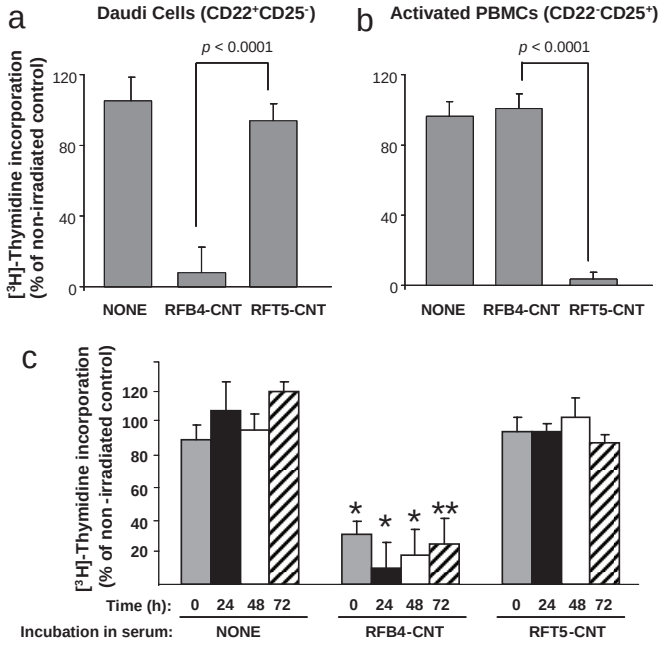

Fig. 5. Ablation of mAb-CNT-coated cells with NIR. One million cells were incubated with saturating concentrations of RFB4-CNTs or RFT5-CNTs. Cells were dispensed into 96-well plates, exposed for 7 min to 808-nm NIR light (5 W/cm^2), pulsed with 1 μCi [^{3}H]thymidine, and harvested 12 h later. The incorporated radioactivity was measured by liquid scintillation counting from triplicate samples. The percentage of radioactivity incorporated by each sample was calculated relative to corresponding nonirradiated sample. (*a*) The specific killing by RFB4-CNTs of Daudi cells using RFT5-CNTs as a negative control ($P < 0.0001$). (*b*) The specific killing of RFT5-CNT on activated PBMCs (>95% T cells) using RFB4-CNTs as a negative control ($P < 0.0001$). Data represent mean ± SD of at least three independent experiments. (*c*) The stability of the mAb-CNTs *in vitro* was determined by incubating them in mouse serum at 37°C for 0, 24, 48, and 72 h. At each time point, the mAb-CNTs were washed with PBS, incubated with Daudi cells, and exposed to NIR light as described above. The activity of the RFB4-CNTs at the different time points remained unchanged. *, $P < 0.0001$; **, $P < 0.05$ for the values obtained at the corresponding time points with RFT5-CNTs. Data represent mean ± SD of three independent experiments.

Discussion

The first critical challenge in the field of targeted CNTs is to create soluble and stable CNTs that retain both the specificity of the targeting moiety and the thermal activity of the CNTs even in serum at physiological temperatures. In this article, we demonstrate that this can be accomplished. Our strategy involved the generation of targeting moieties consisting of mAb-NAs attached to dispersed biotinylated CNTs. The use of B-CNTs and mAb-NAs gives us the flexibility to "assemble" the targeted CNTs by using any cell-binding mAb. Second, the one-step strategy of generating dispersed CNTs by using biotinylated polar lipids has the advantage of preventing subsequent chemical treatments that remove the polar lipids and/or destroy their optical properties. Of equal importance is the specificity of the targeting strategy. Thus, previous studies have demonstrated that folic acid-coated CNTs could be targeted to folate receptor (FR)-positive cells and that NIR light killed the cells (25). Although FR-negative cells were used as a control, CNTs coated with an irrelevant ligand were not. In other studies, rArg-Gly-Asp (RGD)-CNTs were used to deliver adsorbed doxorubicin (29). These CNTs were also evaluated for *in vivo* biodistribution (19), but control peptide-CNTs were not used to demonstrate specificity. Another approach for targeting CNTs to cells is to noncovalently attach mAbs that can be used in photothermal therapy (26) or imaging (30). However, attachment of mAbs by direct adsorption on CNTs involves a potential loss of the targeting function of the mAbs and, indeed in the study cited, specificity controls were not reported, and cell viability studies showed 50% collateral damage by the irrelevant mAb-CNT control after exposure to NIR light (26). In another very elegant study, mAbs were covalently attached to CNTs to deliver radionuclides to cells (28). These studies achieved their goal of killing target cells by radiotherapy and showed both linkage stability and specific targeting. However, because the objective of these studies was not to ablate cells with NIR light, we do not know whether the optical properties of the CNTs were preserved.

Having demonstrated excellent specificity of both targeting and thermal ablation *in vitro*, the next step is to evaluate the pharmacokinetics, biodistribution, toxicity, and activity of these mAb-CNT constructs *in vivo*.

Materials and Methods

Materials. Purified CNTs (HiPco) were purchased from Carbon Nanotechnologies. The polar lipid DSPE-PEG(2000) biotin was purchased from Avanti Polar Lipids. Mouse IgG1 anti-human CD22 (RFB4) and mouse IgG anti-human CD25 (RFT5) were prepared and purified in our laboratory at UT Southwestern Medical Center. Traut's reagent and MBS were purchased from Pierce/Endogen. NA was purchased from Accurate Chemical and Scientific.

Cell Culture. Daudi cells (American Type Culture Collection) were cultured in RPMI 1640 medium (Sigma) containing 1% antibiotic-antimycotic mixture (penicillin/streptomycin/Amphotericin B) (Sigma), 10% heat-inactivated FCS (HyClone), and 2 mM L-glutamine (Sigma) (complete medium). PBMCs from

normal healthy donors were isolated from the fresh heparinized blood by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation. Normal activated $CD25^+$ cells were generated by culturing the PBMCs for 72 h at 1×10^6 cells/ml in complete medium supplemented with 5 μg/ml PHA (Sigma).

CNT Solubilization by Biotinylated Polar Lipids and Characterization. Degassed ultrapure deionized (DI) water was used for all solutions. CNTs (0.3 mg) were suspended in 1 ml of 166 μM DSPE-PEG(2000)-biotin. The mixture was sonicated with a 2-mm probe tip connected to a Branson Sonifier 250 (VWR) for 10 min at a power level of 10 W, with the sample immersed in an ice water bath. To remove excess DSPE-PEG-biotin, samples were washed twice in DI water by centrifugation for 15 min at 90,000 $\times$ *g* at 4°C. The supernatant was discarded, the pellet was resuspended in 1 ml of DI water, and the procedure was repeated. The samples were then centrifuged two times for 10 min at 16,000 $\times$ *g* at room temperature, and the upper 50% of the supernatant containing the B-CNT was recovered. To obtain more concentrated samples, the B-CNT suspension was centrifuged for 60 min at 16,000 $\times$ *g* at 4°C, the supernatant was discarded, and the pellet was resuspended in 0.2 ml of DI water.

Sample concentration was detected by TGA using a Pyris-1 thermal gravimetric analyzer (PerkinElmer) equipped with a high-temperature furnace and sample thermocouple. AFM was performed in air under ambient conditions by using a Digital Instruments Nanoscope III Multimode scanning probe microscope (Veeco Metrology). Images were acquired in the TappingMode by using cantilevers with 0.9 Nm^{-1} force constants as described (32). A dual-beam Lambda 900 UV-Vis-NIR spectrophotometer (PerkinElmer) with a scan speed of 25 nm/min and a 0.4-s integration time was used for absorption spectra. Raman spectroscopy at 633-nm excitation was performed with a LabRAM high-resolution confocal Raman microscope system (Jobin Yvon). Wave number calibration was performed by using the 520.5-cm^{-1} line of a silicon wafer; the spectral resolution was $\approx$1 cm^{-1} as described (33). TEM was performed with a JEOL JEM-1200EX II electron microscope. The B-CNT dispersion was probed with 5-nm gold beads labeled with goat antibiotin (Kirkegaard & Perry Laboratories), and then imaged.

Preparation of mAb-NA Conjugates. To couple the B-CNTs to mAbs, we used a modified protocol (34). Briefly, 10 mg of RFB4 or RFT5 in 1 ml of 0.15 M borate buffer, 0.1 mM EDTA, pH 8.5 were thiolated by incubation for 1 h at room temperature with a 20:1 molar excess of Traut's reagent. After incubation, the reaction was quenched with 0.1 M glycine. In parallel, 10 mg of NA dissolved in 1 ml of 0.01 M PBS, 0.1 mM EDTA, pH 7.4, was activated by 30-min incubation at room temperature by using a 6:1 molar excess of MBS. The unreacted Traut's reagent and MBS were removed by gel filtration on Sephadex G-25 columns in 0.01 M PBS, 0.1 mM EDTA, pH 7.4. The thiolated mAb was conjugated to the activated NA at a molar ratio of 1:2 for 2 h at room temperature with gentle shaking. The resultant conjugate was purified by gel filtration on a Sephacryl S-300 HR column (GE Healthcare) by using 0.1 M PBS, 0.05% Tween-20, pH 7.4. The protein concentration in the purified conjugate was quantified by using the BCA assay (Pierce/Endogen). The size and integrity of the conjugate was analyzed by Western blot. The samples were electrophoresed on a 7.5% nondenaturing polyacrylamide gel and transferred to PVDF membranes (Bio-Rad), probed with HRP-labeled sheep anti-mouse IgG, and visualized by using an enhanced chemiluminescence system (GE Healthcare).

Competition ELISA. NA-coated 96-well plates were blocked with 1% BSA in 0.01 M PBS, 0.05% Tween-20 (PBST) for 1 h. B-CNTs were added to each well together with biotin-labeled HRP and incubated for 1 h. After washing five times with PBST, the substrate ABTS was added, and absorbance was measured at 405 nm. The amount of biotin bound to the CNTs (biotin mmol/μg B-CNT) was calculated by using a standard curve constructed by plotting OD against the biotin concentration (ng/ml) prepared by coincubating increasing amounts of biotin in the presence of a constant amount of HRP-biotin. A similar procedure was used to detect the amount of NA in mAb-NA conjugates.

Preparation of mAb-CNT Conjugate. Fresh mAb-CNTs were prepared immediately before use by mixing B-CNT with mAb-NA in a 1:2 (wt/wt) ratio. The mixture was placed on a rocker for 35 min at room temperature and vortexed gently every 5 min. After coupling, the mixture was centrifuged for 5 min at 16,000 $\times$ *g* at 4°C, the supernatant containing unreacted mAb-NA was discarded, and the pellet was resuspended in 40 μl of PBS for every 3.6 μg of B-CNT and used immediately.

Binding of mAb-CNTs to Target Cells. One million Daudi cells or PHA-activated PBMCs (>95% $CD25^+$ cells) were incubated with the mAb-CNTs for 20 min at 4°C in PBS. Cells were washed two times with ice-cold PBS and then incubated with either PE-SA (Jackson ImmunoResearch) or FITC-GAMIg (Kirkegaard & Perry Laboratories) for 20 min at 4°C. The cells were washed two times with ice-cold PBS and resuspended in 0.5 ml of PBS, and the bound fluorescence was analyzed on a FACScan (Becton Dickinson).

Determination of the Amount of B-CNTs Bound per Cell. One million Daudi cells were incubated with saturating amounts of RFB4-NA or RFT5-NA for 15 min at 4°C in PBS. Cells were washed two times with ice-cold PBS, incubated with incremental amounts of B-CNT for 20 min at 4°C in PBS, and then washed two times with ice-cold PBS. The amount of B-CNT bound to cells was determined by measuring the absorbance at 808 nm of the B-CNT suspension before and after incubation with Daudi cells. The amount of B-CNT bound per cell was determined by using the extinction coefficient [$\varepsilon^{0.1\%} = 25$ $(mg/ml)^{-1}$] calculated from the linear fit (Beer-Lambert law) of absorbance at 808 nm versus the B-CNT concentration.

Ablation of mAb-CNT-Coated Cells with NIR Light. One million cells were incubated with 40 μl of the mAb-CNTs in PBS for 20 min at 4°C. Cells were washed three times with ice-cold PBS, and then 10^5 cells were dispensed in triplicate wells in a 96-well plate in 200 μl of complete medium. The cells were exposed to continuous NIR light by using a FAP-Sys-30W 805- to 811-nm laser system (Coherent) for 7 min at 5 W/cm^2. Cell death was assessed by pulsing the cells for the next 12 h with 1 μCi [^{3}H]thymidine per well, and the incorporated radioactivity was measured by liquid scintillation counting. The incorporated radioactivity for each sample was calculated relative to the corresponding nonirradiated samples. For functional stability, mAb-CNTs, prepared as described above, were suspended in 0.1 ml of mouse serum (Sigma) and incubated at 37°C for 0–72 h. At each time point, the suspension was washed with ice-cold PBS, and the pellet was resuspended in 40 μl of PBS. The ablation of mAb-CNT-coated Daudi cells with NIR light was tested as described above.

Statistics. Data were analyzed by using Student's *t* test. Values are given as mean $\pm$ SD. $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS. We thank Dr. Gregg R. Dieckmann (University of Texas, Dallas) for use of the sonicator and centrifuge; Hadi N. Yehia for help during the initial stages of the project; Vicky Poenitzsch for assistance with the AFM images; Kate Walker, Meredith Daigrepont, and Jonathan Belew for assistance with elemental analyses; and Drs. Peter Wilk, Robert Stirbl, Jimmy Xu, and Malcolm Snead for unwavering support, ideas, and interactions. This work was supported by The Cancer Immunobiology Center, University of Texas Southwestern Medical Center (E.S.V., R.M., and P.C.), Robert A. Welch Foundation Grants AT-1364 (to P.P.) AT-1326 (to I.H.M.), the Department of Defense (to E.S.V. and R.K.D.) and the Center for Applied Biology at the University of Texas, Dallas. N.S.Z. and A.D.-E.S. were summer students supported by the Summer Undergraduate Research Fellowship program at the University of Texas Southwestern Medical Center.

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THE UNIVERSITY OF TEXAS DALLAS
INTELLECTUAL PROPERTY QUESTIONNAIRE

DESCRIPTION OF THE INVENTION

Descriptive Title of Invention:

Method for Measuring Carbon Nanotubes Taken-Up by a Plurality of Living Cells

Who are the individuals that contributed to the conception of the invention (attach Contributor Information Page for each):

Corresponding Contributor: Rockford K. Draper

Other Contributors: Paul Pantano, Ru-Hung Wang, and Carole A. Mikoryak

INTELLECTUAL PROPERTY DESCRIPTION

(Attach separate pages, if necessary)

Briefly summarize the invention, its use and purpose: The following invention describes a new analytical method that can provide a rapid, inexpensive, label-free measurement of the concentration of all carbon nanotube (CNT) structures in a liquid sample; the method requires only a gel-electrophoresis unit, a flatbed scanner, and basic image analysis software.

What particular features of the invention are unusual? While the "Preferred Embodiment" section demonstrates how the invention can be used to determine how many CNTs are taken up by living cells, the invention can alternatively be used to determine CNT concentrations from any biological matrix, and additionally, any industrial process or waste stream. Furthermore, the invention is not limited to the detection of nanotubes; it should find applicability in determining the concentration of almost any nanoparticle type (e.g., fullerenes, quantum dots, inorganic or metallic nanospheres and nanorods, etc.) present in a liquid sample.

How does it differ from present technology? In brief, the new method differs from the current technology in that it does not require sophisticated and expensive imaging/spectroscopic instrumentation to measure CNTs. Instead, the invention is a separation and detection methodology that uses a common laboratory apparatus – a gel electrophoresis unit, and a common office supply – a flatbed scanner. This key difference is what permits the invention to be considered affordable to any individual laboratory unit.

What problem(s) does it solve? Improved analytical methods for identifying nanomaterials in biological systems is a specific federal priority for advancing nanotechnology as cited by the National Nanotechnology initiative consortium in winter of 2008. Indeed, the success of almost all biomedical applications of CNTs to living cells, ranging from intracellular sensors to chemotherapeutic agents, will ultimately depend on how many CNTs are taken up by cells. Statistically meaningful measures of the amounts of CNTs inside cells are therefore required to evaluate the effectiveness of these applications. Current methods to detect CNTs inside cells possess one or more of the following disadvantages: they are unable to detect both metallic and semi-conducting CNT structures, they are unable to analyze CNTs in living cells, they are not suited to analyze a large population of cells in a timely fashion, and/or they require the attachment of a reporter label to the CNT. The invention represents the first rapid, affordable method that can serve this purpose.

What advantages over current technologies does it possess? To our knowledge, this affordable invention represents the only label-free methodology that can rapidly detect CNT structures from microscopic samples such as living cells and macroscopic samples such as industrial process streams,

Preferred Embodiment

In the following demonstrative example, we detail the extraction and quantitative detection of the levels of single-walled carbon nanotubes (SWNTs) taken-up by a population of biological cells.

I. Preparation and characterization of the dispersed SWNT sample.

For cell uptake studies, various concentrations of bovine serum albumin (BSA)-coated SWNTs were prepared by sonicating (10 min at 0 °C with a tip sonicator, 10W) 1 mg of SWNTs (CoMoCAT, from Southwest Nanotechnologies, Inc.) in 1 mL of various concentrations of aqueous BSA. Each dispersion was centrifuged once at 16,000x g for 2 min, the supernatant removed, and centrifuged again for 2 min at the same speed. The final supernatants (i.e., BSA-SWNTs) were very dark, indicating dispersion of the black SWNTs (Figure 1). The absorption spectra of the BSA-SWNT dispersions shown in Figure 2 indicate that SWNTs dispersed in BSA retain their optical transitions between van Hove singularities in the electronic density of states, and that the concentration of the debundled SWNTs increases with increasing BSA concentrations (correlation coefficient = 0.9925). Elemental analysis of BSA-SWNTs found background levels of molybdenum and cobalt, catalysts used in making this type of SWNT, indicating that >99% of these contaminants were removed by our dispersion preparation protocol.

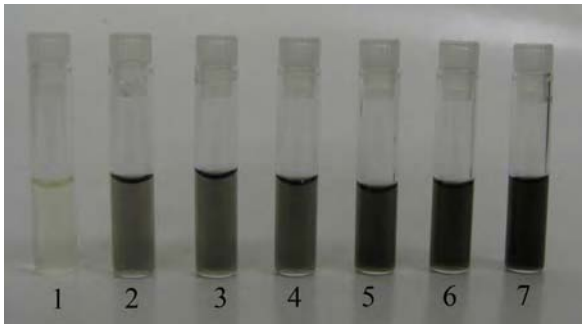


Figure 1. Photograph of vials containing CoMoCAT SWNT dispersions prepared in aqueous BSA solutions. The concentration of BSA varied from **(1)** 0 mg/mL, **(2)** 12.5 mg/mL, **(3)** 25 mg/mL, **(4)** 50 mg/mL, **(5)** 100 mg/mL, **(6)** 150 mg/mL, to **(7)** 200 mg/mL.

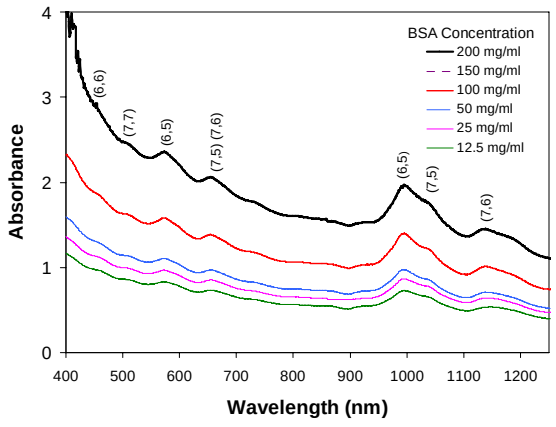


Figure 2. Background-corrected absorption spectrum of the CoMoCAT BSA-SWNT dispersions shown in Figure 1. The main metallic and semi-conducting SWNT structures are denoted by their (*n,m*) chiral indices.

BSA-SWNTs were further characterized using Raman spectroscopy, which revealed a strong "G" line in the 1550-1610 cm^{-1} region, and other lines typical for SWNTs (Figure 3). SWNTs in the dispersions increased with increasing BSA concentrations, indicated by the increasing "G" line intensities (correlation coefficient = 0.9232). Control samples of BSA without SWNTs did not display detectable resonances under these operating conditions (data not shown).

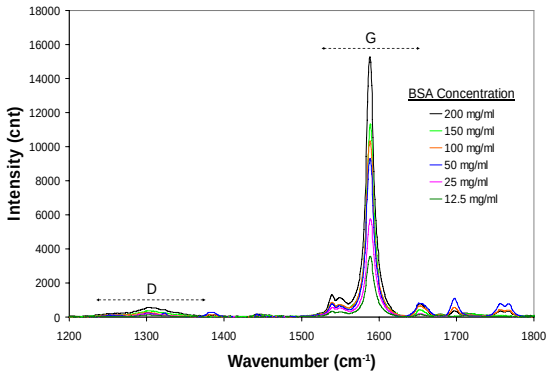


Figure 3. Raman spectra (633 nm laser excitation) acquired from CoMoCAT BSA-SWNT dispersions prepared in BSA solutions of various concentrations; all spectra were normalized to the same intensity scale.

II. Detection of SWNTs in BSA-SWNT samples by SDS-PAGE gel electrophoresis.

The BSA-SWNT dispersions characterized above were analyzed by SDS-PAGE gel electrophoresis to demonstrate the linearity and specificity of the invention. As shown in Figure 4, individual BSA-SWNT dispersions were run in separate lanes of a conventional SDS polyacrylamide gel. In brief, both proteins and SWNTs bind SDS and migrate towards the cathode because they have a negative charge from the sulfate on the SDS. Unlike most proteins, however, the SWNTs are too large to enter the gel and accumulate in a band at the bottom of the sample loading wells. The accumulation of the black SWNTs at this interface also considerably concentrates the SWNTs, which increases the sensitivity of detection.

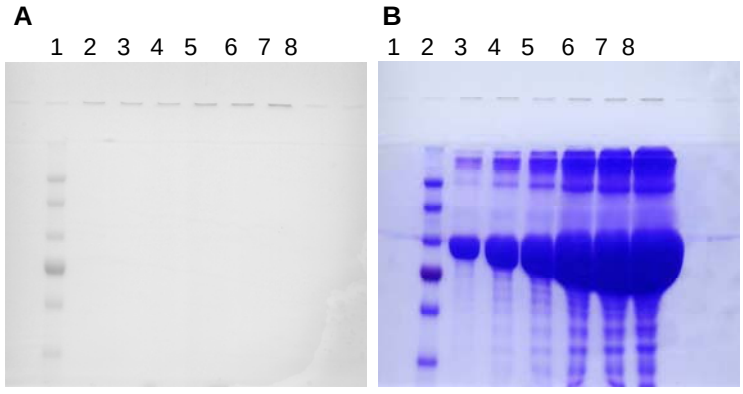


Figure 4. SDS-PAGE gel electrophoresis of BSA-SWNTs and controls at 100 mV for 2h. **(A)** and **(B)** demonstrate separation of SWNTs from protein components after electrophoresis using digital scans of a 4% stacking 10% resolving 1.5 mm thickness mini gel before **(A)** and after **(B)** proteins stained with 0.5 % Commassie Blue. Lane (**1**) is blank; lane (**2**) is pre-stained protein molecular weight markers; lanes (**3**) to (**8**) are 1 uL of SWNT dispersions prepared in various BSA concentrations: (**3**) 12.5 mg/mL, (**4**) 25 mg/mL, (**5**) 50 mg/mL, (**6**) 100 mg/mL, (**7**) 150 mg/mL, (**8**) 200 mg/mL.

The black SWNTs are quantified at the interface after scanning the gel with a digital imager, followed by measuring the pixel intensities of the bands with ImageQuant software. Figure 5A shows the pixel intensities of the 6 dark bands shown in the top row of the gel in Figure 4A. The band intensities demonstrate that the signal is directly proportional to the material applied to the gel (correlation coefficient = 0.9625) and that the signal is linear with concentration over a 20-fold range. When the dark bands are scanned using the Raman microprobe, prominent G lines are observed, demonstrating that the material in the bands are SWNTs (Figure 5B). Additionally, the linearity of G-line intensities from the various BSA-SWNT dispersions (correlation coefficient = 0.9766) agree favorably with those determined by the gel electrophoresis/scanner detection method (Figure 5B-inset).

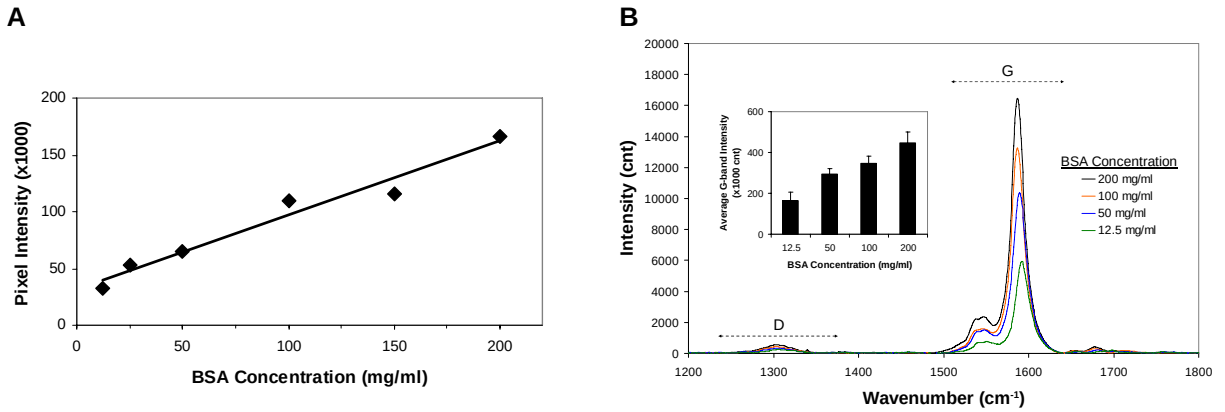


Figure 5. Comparison of **(A)** pixel intensities of the digital image and **(B)** Raman spectra of SWNT bands after electrophoresis in a SDS-PAGE gel as a function of BSA concentration used to prepare the BSA-SWNT dispersions. Raman spectra (633 nm laser excitation) acquired from CoMoCAT BSA-SWNT dispersions prepared in BSA solutions of various concentrations; all spectra were normalized to the same intensity scale.

Further evidence demonstrating that the dark material observed in the dried gel comprises SWNTs is provided by a series of experiments whereby multiple regions above and below the interface of the gel were analysed by microprobe Raman spectroscopy with ~5 μm lateral resolution. In these experiments, the 633-nm laser beam was first focused on the center of the SWNT band near the bottom of the well. As shown in Figure 6, spectra were acquired from this region and at various spots along the length of the lane, above and below the SWNT band. The resulting vertical profile of G-line intensities clearly indicates that SWNT material is detected only in the band at the interface of the sample and the gel (i.e., loading well).

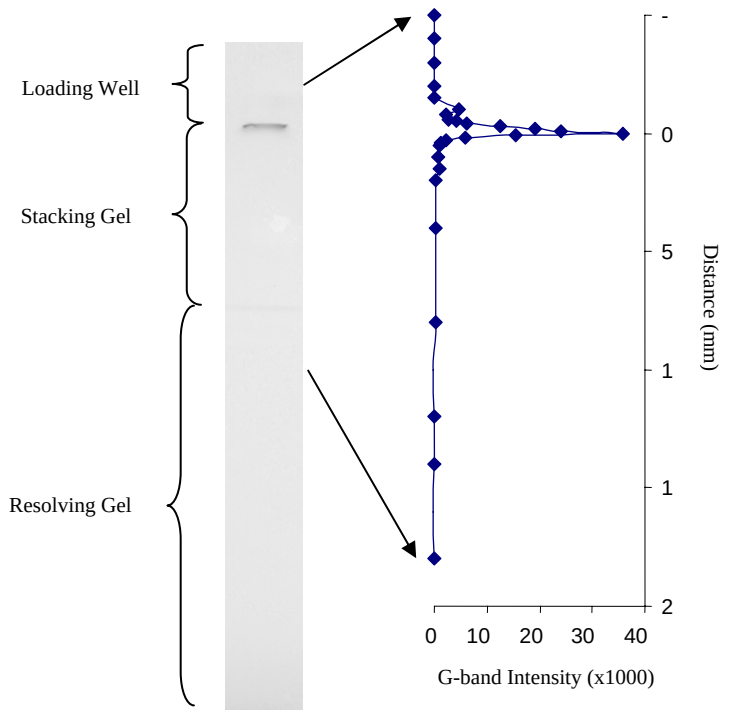


Figure 6. Raman spectra G-line intensities as a function of distance from the bottom of the sample loading well within a lane of a SDS-PAGE gel. Each G-line intensity was calculated by integrating the intensities in the 1510 – 1630 cm^{-1} region.

III. Raman measurements of the uptake of SWNTs by living cells.

The following section briefly reviews our published work on measuring the uptake of peptide- and protein-coated SWNTs by cells,[Chin et al., 2007][Yehia et al., 2007] with the emphasis being on the experimental designs and controls required to properly classify whether SWNTs are taken-up by cells.

HeLa cells were incubated at 37 °C for 22 h in medium containing peptide-coated SWNTs. The cells were then washed extensively and analyzed by microprobe Raman spectroscopy to determine whether the SWNTs were inside cells by detection of their distinctive G-line signature at 1590 cm^{-1}. Figure 7 shows typical results with one cell out of numerous cells that were analyzed. When the area probed was outside the cell, the Raman G line signal was negligible, but when either the cell cytoplasm or nuclear areas were probed, the signal was present at 1590 cm^{-1} (Figure 7). Cells that were not exposed to SWNTs had no G line (data not shown).

Chin S-F, Baughman RH, B DA, Dieckmann GR, Draper RK, Mikoryak C, Musselman IH, Zorbas-Poenitzsch V, Pantano P. Amphiphilic helical peptide enhances uptake of single-walled carbon nanotubes by living cells. Exp Biol Med 232:1236-1244, 2007.

Yehia HN, Draper RK, Mikoryak C, Walker EK, Bajaj P, Musselman IH, Daigrepont MC, Dieckmann GR, Pantano P. Single-walled carbon nanotube interactions with HeLa cells. J Nanobiotechnology 5: 8-58, 2007.

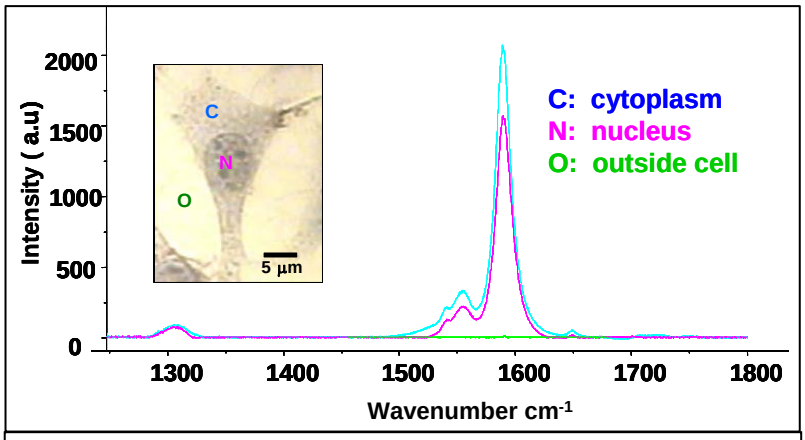


Figure 7. Raman spectra from a single HeLa cell following exposure to dispersed SWNTs. The inset is a micrograph of the cell studied showing the locations that were analyzed.

The signal emanating from SWNT-treated cells could be from nanotubes inside the cells, or stuck to the exterior cell surface. If the signal was from inside the cells, most likely the result of active uptake, such as endocytosis, then the signal should be absent in cells exposed to the SWNTs at 4 °C where energy-dependent uptake ceases. Figure 8 compares the Raman spectra from a cell exposed to SWNTs at 37 °C and 4 °C for 22 h, washed extensively, and prepared for Raman microprobe analysis. There was essentially no SWNT signal from this cell, or others analyzed at 4 °C, evidence that the Raman signal from cells exposed to SWNTs is from nanotubes that required metabolic activity to accumulate in cells. This result also shows that any SWNTs adhering to the outside of cells at 4 °C were washed away, arguing that the Raman signal from cells at 37 °C is due to nanotubes inside the cells, not adhering to the outside.

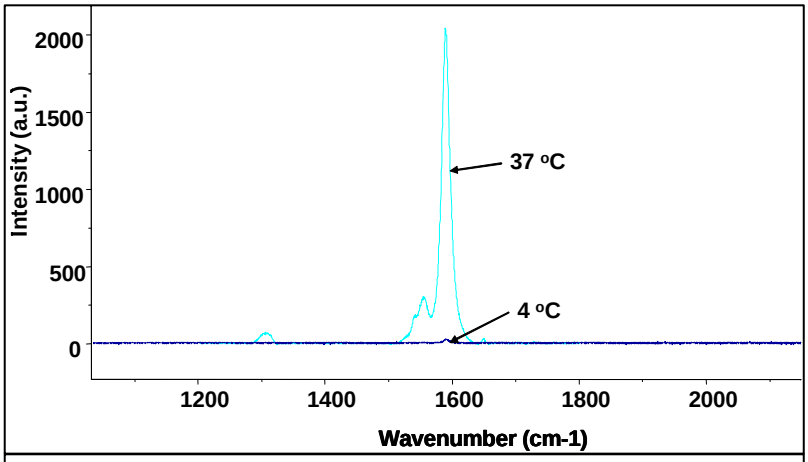
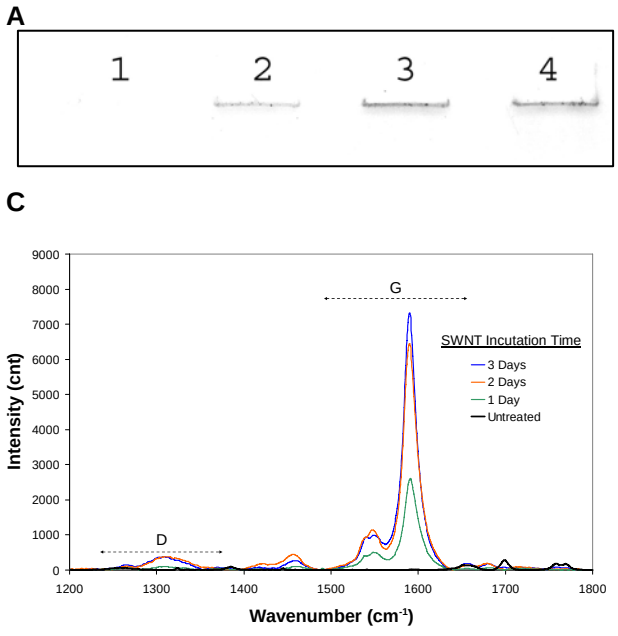


Figure 8. Raman spectra of HeLa cells exposed to dispersed SWNTs at either 37 or 4 °C.

IV. Electrophoresis/scanner measurements of the uptake of SWNTs by living cells.

The experimental designs and controls for the single cell Raman measurements described in the preceding section were used to measure the bulk uptake of SWNTs by cell populations using our novel electrophoresis/scanner methodology. In the following examples, bulk SWNT uptake levels were measured as a function of cell incubation time and temperature, and BSA-SWNT concentration. In all cases, after NRK cells in culture were incubated in BSA-SWNTs and thoroughly washed, cell-associated SWNTs (either on the cell surface or within the cells) were extracted by treatment with 200 µl of extraction solution. The extraction solution contained 1% SDS, 1mM $MgCl_2$, 1mM $CaCl_2$, and 20 ug of 1mg/mL DNase I per mL. The DNase digests DNA released from cells by SDS even in the presence of the SDS and reduces the viscosity. The 1 mM $MgCl_2$ and $CaCl_2$ are metal ions required by the DNase. Total cellular protein content in the cell lysate was determined by BCA protein assay. Cell lysate samples of equal protein content were then electrophoresed by standard 4% stacking 10% resolving SDS-PAGE for 2 hours at 100 volts. The pixel intensities of the SWNT bands were measured by imaging using a flatbed scanner and quantified using ImageQuant software as described above.

In the first example, NRK cells in culture were incubated in BSA-SWNTs (constant concentration) continuously for 1, 2, or 3 days. As expected, Figure 9 shows that the amount of SWNTs taken up by NRK cells increases as a function of incubation time (correlation coefficient = 0.9922), and that no appreciable signal was generated for control cells not exposed to BSA-SWNTs. Finally, as shown in Figure 9c, microprobe Raman spectroscopy verified the presence of SWNTs in the band by the presence of a strong G-line signature.



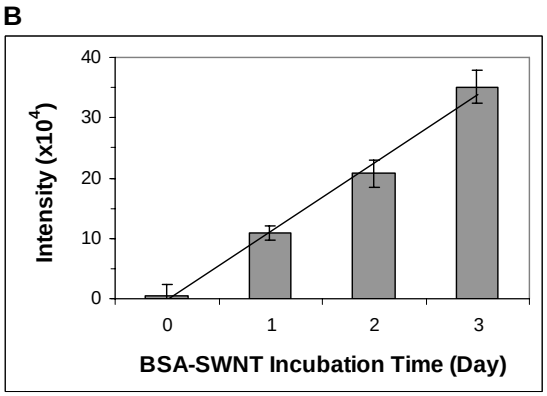


Figure 9. **(A)** A digital image showing the SWNT band region of the SDS-PAGE gel after electrophoresis. Lane (**1**) is 3 days incubation in media contains no SWNT dispersion; lanes (**2**) to (**4**) are cells incubated in media containing BSA-SWNT for 1, 2, or 3 days, respectively. **(B)** Relative pixel intensity of SWNT band as indicative of SWNT content in cell lysate as a function of incubation time. The average and standard deviation values were determined from three independent experiments. **(C)** Raman spectra acquired on the dried gel using a 50x objective with laser excitation at 633 nm focused

on SWNT band areas. The representative spectra were obtained by merging three spectra acquired from the dark band area in each lane.

In the second example, NRK cells in culture were incubated continuously for 3 days in BSA-SWNT dispersions of differing concentrations. As expected, Figure 10 shows that the amount of SWNTs taken up by NRK cells increases as a function of SWNT concentration in the BSA-SWNT dispersions (correlation coefficient = 0.9811), and that no appreciable signal was generated for control cells not exposed to BSA-SWNTs.

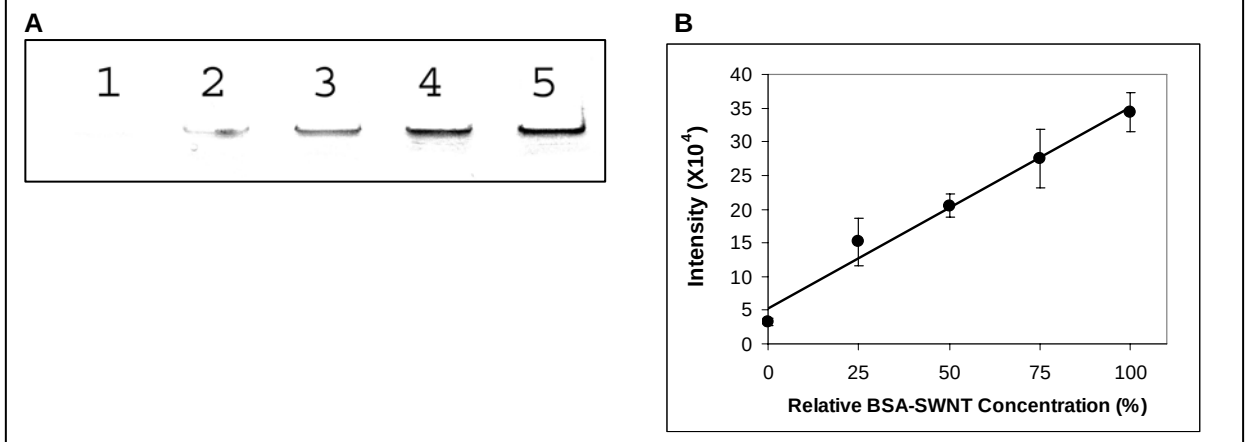


Figure 10. (A) A digital image scanning shows the SWNT bands region of the SDS-PAGE gel after electrophoresis. Lane (**1**) is control media contains no SWNT dispersion; lane (**2**) is 25% SWNT dispersion; lane (**3**) is 50% SWNT dispersion; lane (**4**) is 75% SWNT dispersion; lane (**5**) is media with undiluted SWNT dispersion. **(B)** Relative pixel intensity of SWNT band as indicative of SWNT content in cell lysates after 3 days uptake. The average and standard deviation values were determined from three independent experiments.

In the third example, NRK cells in culture were incubated continuously for 1 day in BSA-SWNTs (constant concentration) at two temperatures. As expected, Figure 11 shows that there was essentially no SWNT signal from cells analyzed at 4 °C vs. cells analyzed at 37 °C. This result suggests that any SWNTs adhering to the surface of cells were washed away, and that the SWNT signal detected is from SWNTs that accumulate inside cells, owing to the cellular metabolic activity that occurs at 37 °C but not at 4 °C.

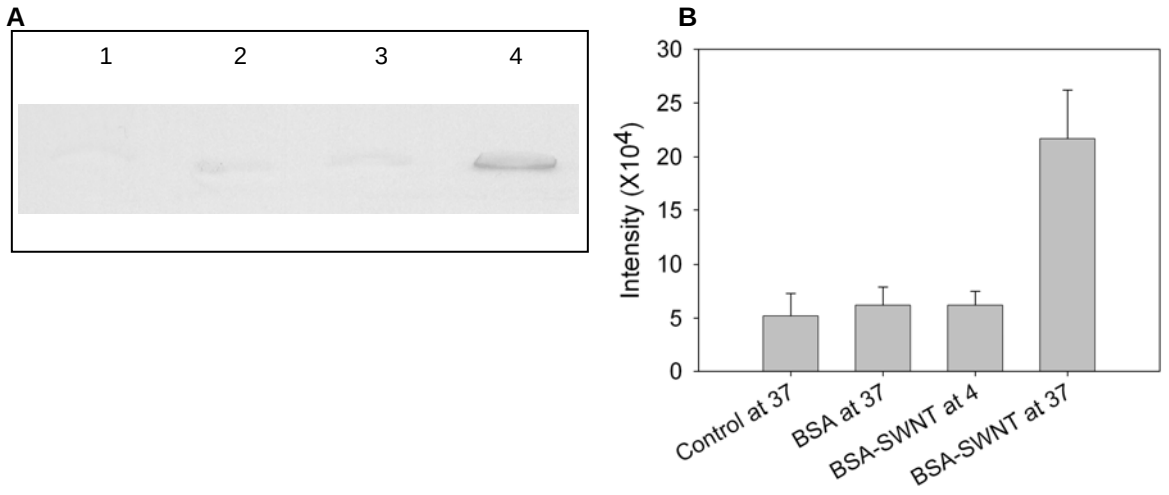


Figure 11. **(A)** A representative digital image scanning shows the SWNT bands region of the SDS-PAGE gel after electrophoresis. Lane (**1**) is untreated control cells at 37^{o}C; lanes (**2**) to (**4**) are cells incubated in media contains (**2**) BSA at 37^{o}C, (**3**) BSA-SWNT at 4^{o}C, and (**4**) BSA-SWNT at 37^{o}C for 24 hours. **(B)** Relative pixel intensity of SWNT band as indicative of SWNT content in cell lysate samples. The average and stander deviation values were obtained from three independent experiments.

VERY IMPORTANT: If material, biological or non-biological, is incorporated or was used in the research leading to the invention, was this material obtained from some source other than your laboratory? (Use additional sheets if necessary)(circle):

Yes (No) If yes, what is material(s)? _____
What is the source of the material(s)?
UTD laboratory _____
Company _____
Other Institution _____
Other (explain) _____

Yes (No) Was this material(s) covered by a Material Transfer Agreement, Sponsored Research Agreement, Collaboration Agreement, or any other type of agreement that restricts the use of this material(s)?

If yes, please attach a copy of the agreement pertaining to the use of the material.

Please categorize the potential uses of your invention below by circling all anticipated uses:

Service	(Research Reagent)
(Product-Therapeutic)	(Drug Identification)
(Product-Device)	(Diagnostic Test)
(Product-Method)	Other: _____

What companies do you believe would be interested in commercializing the invention?

Medical Nanotechnologies Inc. Nanospectra Biosciences Inc.

ThermMed LLC Unidym Inc.

Texas Instruments and other semiconductor fabrication companies

Have you worked with/been approached by any companies regarding the invention (circle): (Yes) No
If Yes, which companies?

Medical Nanotechnologies Inc.

CONTRIBUTOR INFORMATION

Attach one page for each contributor named on the first page.

Contributor's Name: Draper (Last) Rockford (First) K. (MI) Ph.D. (Degree)

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If a patent application for the intellectual property disclosed above is filed in the U.S. Patent and Trademark Office, the inventor(s) will be required to execute an oath or declaration affirming, among other things, that he/she/they is/are the original and first inventor(s) of the subject matter claimed in the application. ("Original" means derived from any source or persons other than the person or persons named as the inventorship entity.) I/We confirm that the subject matter disclosed herein is original to me/us.

Contributor's Signature: _____

Typed/Printed Name: Rockford K. Draper Date: _____

Please attach a copy of your current C.V.

CONTRIBUTOR INFORMATION

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(If yes, name of employer: _____)

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If a patent application for the intellectual property disclosed above is filed in the U.S. Patent and Trademark Office, the inventor(s) will be required to execute an oath or declaration affirming, among other things, that he/she/they is/are the original and first inventor(s) of the subject matter claimed in the application. ("Original" means derived from any source or persons other than the person or persons named as the inventorship entity.) I/We confirm that the subject matter disclosed herein is original to me/us.

Contributor's Signature: _____

Typed/Printed Name: Paul Pantano Date: _____

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CONTRIBUTOR INFORMATION

Attach one page for each contributor named on the first page.

Contributor's Name: Wang (Last) Ru-Hung (First) (MI) Ph.D. (Degree)

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% of time employed at UT: 100% Other employment (circle one)? Yes (No)

(If yes, name of employer: _____)

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Home Phone #: (214) 350-2891

If a patent application for the intellectual property disclosed above is filed in the U.S. Patent and Trademark Office, the inventor(s) will be required to execute an oath or declaration affirming, among other things, that he/she/they is/are the original and first inventor(s) of the subject matter claimed in the application. ("Original" means derived from any source or persons other than the person or persons named as the inventorship entity.) I/We confirm that the subject matter disclosed herein is original to me/us.

Contributor's Signature: _____

Typed/Printed Name: Ru-Hung Wang Date: _____

Please attach a copy of your current C.V.

CONTRIBUTOR INFORMATION

Attach one page for each contributor named on the first page.

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% of time employed at UT: 75% Other employment (circle one)? Yes (No)

(If yes, name of employer: _____)

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If a patent application for the intellectual property disclosed above is filed in the U.S. Patent and Trademark Office, the inventor(s) will be required to execute an oath or declaration affirming, among other things, that he/she/they is/are the original and first inventor(s) of the subject matter claimed in the application. ("Original" means derived from any source or persons other than the person or persons named as the inventorship entity.) I/We confirm that the subject matter disclosed herein is original to me/us.

Contributor's Signature: _____

Typed/Printed Name: Carole A. Mikoryak Date: _____

Please attach a copy of your current C.V.

PUBLICATIONS AND/OR ORAL DISCLOSURES

Identify any disclosures, either written or oral, of the intellectual property made or to be made to others. ATTACH COPY OF PUBLICATION(S) OR DRAFT THEREOF. Please also attach: (1) any additional publications which will give background information for the IPAC committee; and (2) the most relevant articles, journals, or abstracts of another author.

Publication or oral disclosure (1)

Title: Characterizing Single-Walled Carbon Nanotube Production Lots and Measuring the Uptake of Purified SWNTs by Living Cells nanoTX'07 Nanotechnology Conference and Trade Show

Authors: Paul Pantano

Type of disclosure (circle): Written (Oral) Other: _____

If written (circle): Journal Abstract Poster Other: _____

Publication Date: 4 October 2007 Location: Dallas, Texas

Publication category (circle): (Public) Confidential Internal Other _____

If confidential (circle): Confidentiality agreement complete
Letter
Oral Other: _____

FINANCIAL RESOURCES USED/OBLIGATIONS

Please circle one answer for each of the following questions. If yes to any below, please fill in grant/contract information as listed.

Was development of this intellectual property aided in any way by a grant or contract? (Yes) No

Is this intellectual property the subject of a currently pending grant or contract proposal? Yes (No)

To your knowledge, is this intellectual property encumbered or obligated to any third party? Yes (No)

Grant/Contract/Sponsor (1)

Entity Name: The Robert A. Welch Foundation

Type (circle): NIH DARPA DOD Other Federal Industry
Non-Profit Private Donor Other: Private Foundation

Title of Project: Fundamental Research Grant / "Intracellular Molecular-Nanotube Complexes"

Briefly describe purpose: This research project has been designed to advance medical applications of nanotubes. Fundamental projects include investigating the intracellular fate and biological response of functionalized nanotubes and elucidating the molecular mechanisms of nanotube biocompatibility.

UTD Acct #: 643205 Grant or Contract #: AT-1364

Name of project manager for the contract at DARPA or DOD:

Grant/Contract/Sponsor (2)

Entity Name: US Army MED RESEARCH AND MATERIEL COM

Type (circle): NIH DARPA (DOD) Other Federal Industry
Non-Profit Private Donor Other:

Title of Project: Interdisciplinary Studies on the Combat Readiness and Health Issues Faced by

Military Personnel: Project 3, Carbon Nanotubes in Cancer.

Briefly describe purpose: To use targeted carbon nanotubes in cancer chemotherapy

UTD Acct #: 632087 Grant or Contract #: Dr. Julie Wilberding

Name of the project manager for the contract at DARPA or DOD:

UT D

THE UNIVERSITY OF TEXAS AT DALLAS

BOX 830688 MP15 RICHARDSON, TEXAS 75083-0688
(972) 883-4579 FAX (972) 883-2310

OFFICE OF RESEARCH COMPLIANCE

Date: **20 June 2007**

To: **John Hart, Jr., M.D.**
Sandra B. Chapman, Ph.D.
Center for BrainHealth

From: **Sanaz Okhovat**
Assistant Director
Office of Research Compliance

Re: **Approval of IRB File Number: 07-27**
Repetitive Transcranial Magnetic Stimulation (rTMS) to Temporarily Reduce Over-Arousal to Emotional Stimuli

This letter is notification of Approval of the research project IRB File 07-27. IRB approval of this research begins as of **20 June 2007** and ends on **19 June 2008.**

Please note that this study has been approved for 45 participants. You must obtain IRB approval prior to increasing the number of participants in this study.

The IRB requires that you report as soon as possible any unexpected adverse events (including non-serious and serious events) that occur during the study. If the research is expected to continue beyond 12 months, you must request Continuing Review and re-approval of the project least 6 weeks prior to the date of expiration date noted above.

If you plan to change the research project (number of participants, title, procedure, payment, consent form, etc.), you must submit a request detailing the proposed changes and receive IRB approval before the changes are implemented except when prompt changes are necessary to eliminate apparent and immediate hazards to the participants.

The IRB requires that all personnel who interact with research participants or who have access to research data be trained in research ethics and practices concerned with the protection of the welfare and rights of research participants. These ethical principles are outlined in the Belmont Report.

All investigators and key personnel involved with this protocol must have documented training with this office. The training can be found at http://www.utdallas.edu/research/compliance/irb/training.html

If you have any questions related to this approval, you may me phone at 972-883-4579 or by email at sanaz.okhovat@utdallas.edu.

UTD IRB Approval: (Date)
UTD IRB Expiration: (Date)

Appendix B

University of Texas at Dallas

CONSENT TO PARTICIPATE IN RESEARCH

Title of Research Project: Repetitive Transcranial Magnetic Stimulation (rTMS) to Temporarily Reduce Over-Arousal to Emotional Stimuli

Investigators:	**Contact Number**
John Hart, Jr., M.D.	972-883-3212
Sandi Chapman, Ph.D.	972-883-3404
Frank Andrew Kozell, M.D.	214-648-5233
George Kondraske, Ph.D.	817-272-2335

Purpose: The purpose of this study is to provide information regarding the use of rTMS to reduce a hyperarousal response in specific individuals.

Description of Project: This research is being done because hyperarousability can lead to impaired cognitive performance in individuals when it is present to a significant degree. In some diseases where there is a distinct trigger for hyperarousability (for example, phobias) it is unknown what part of the brain is involved in this over-arousal and there are not always effective means of treatment. Treatment for these diseases does not necessarily reduce the symptom of hyperarousability. This has been particularly found where a simple stimulus or a stimulus associated with a traumatic event is the trigger for hyperarousability. Hyperarousability provides significant debilitation as well as a heightened sense of over-vigilance to potential stimulus triggers. In addition, normal individuals with over-arousal to threatening or emotional stimuli may perform less than optimally in terms of attention and response if presently with such a stimulus (for example, a young individual in combat).

You are invited to participate in this research because you are between the ages of 21 and 35 and either have a heightened arousal to emotional stimuli or a control subject. You are being asked to volunteer for a research study that evaluates an experimental device to determine if it can temporarily reduce the over-response to threatening stimuli of this disorder. Repetitive Transcranial Magnetic Stimulation (rTMS) is an experimental procedure, where a device called a "stimulator" provides electrical energy to a "magnetic coil" that delivers a magnetic field. When the coil is placed against the scalp on the right front region

of the head, the magnetic field is focused to a region of the brain that is thought to be involved in causing this over-responsiveness to certain objects. This study is intended to test the theory that the electromagnetic field created by the coil may affect this region of the brain in a way that may temporarily block the symptoms of over-arousal to threatening pictures (pictures of lion growling, soldier in the jungle, etc.) Changes in magnetic fields during rTMS (Repetitive Transcranial Magnetic Stimulation) administration induce electrical currents which may affect brain activity and function. The purpose of this study is to obtain information regarding the use of rTMS to determine if rTMS results in a temporary change in this over-arousal response.

You are included in this study because you are a normal control subject or a subject with a medical condition which is associated at times with a heightened arousal to emotional stimuli.

Screening: If you agree to take part and have signed this form, at the first visit, you will complete an examination to determine whether or not you qualify for enrollment in this trial. This first (B1) visit will take about three hours. During this time, you will be answering a series of questions that will judge your mood, presence of a diagnosis of psychiatric illnesses, mental functioning, and quality of life. You cannot be included in this study if you have Post-Traumatic Stress Disorder or phobias to the type of emotional stimuli used in this study, schizophrenia, bipolar disease, dissociative disorders, gender identity disorder, personality disorders, and/or substance abuse.

You will also be asked about your medical history. It is very important that you tell the doctor if you have any history of head injury, epilepsy, seizures of any kind, brain tumor, cancer, and/or stroke. You will then receive a complete medical examination, provide information about medications you have taken, or are currently taking. A safety interview will be conducted to be sure that you will have no increased risk from undergoing assessment with the magnetic stimulator (e.g., metal implants, nerve stimulator devices). A urine drug screen will be done to determine that you are not currently taking any prohibited substances. If you are a woman who is capable of bearing children, a urine pregnancy test will be administered to determine if you are pregnant (pregnant women are not allowed to be involved in this study). This first visit will tell us if you are able to take part in the study.

Participants
Initials: _____

Pre-rTMS Human Performance Capacity Testing: If you agree to participate in this study and meet all eligibility items, you will be administered a set of brief performance tests shortly after the screening process is completed. These tests measure the speed at which you process information and your ability to carry out simple tasks requiring coordination. You will be asked to respond to lights as fast as you can by moving your hand from one switch to another switch on a special board interfaced to a computer. In another test, you will be asked to remember sequences of lights and respond by touching sensors corresponding to the light sequence. You will also use a reach-and-tap motion with your arms to alternate between two targets as fast and as accurately as you can. A final test requires you to stand on one leg as steady as you can for 15 seconds. Each of these tasks will be repeated several times. The entire process will take about 45 minutes. There will be brief rest periods between different tests.

If you find that any of these tasks are disturbing to you, inform the experimenter and the testing will be stopped. If for any reason you wish to discontinue the testing that day, tell us and we will end the testing session. You may withdraw from this study at anytime.

Participants
Initials: _____

Pre-rTMS Brain Wave Response Testing: In this study you will be asked to view images and hear sounds and make judgments about the sounds and images. The images will consist of objects and scenes that will be either neutral or emotionally arousing pictures. The emotionally arousing pictures represent threatening animals or combat scenes. The sounds will consist of tones and environmental sounds. Some of the sounds represent threatening animals or weapons.

During the tasks, a cap of sensors will be places on your head to record your brain waves while you are doing these tasks. The cap is similar to a loose nylon swim cap with 64-128 small sensors lying in it. The cap will be placed on your head and then a small amount of a salt water mixture will be added to each sensor from the outside of the cap. Small video cameras on a nearby table will track where your eyes are looking at the pictures. The entire experiment and placement of the cap and eye tracker will take approximately 60 minutes. At the completion of this section the eye-tracking device and electrode cap will be removed.

If you find that the pictures or sounds are too disturbing for you, inform the experimenter and the testing will be stopped. If for any reason you wish to discontinue the testing that day, tell us and we will end the testing session. You may withdraw from this study at anytime.

Participants
Initials: _____

rTMS Administration and Post-rTMS Testing: After completion of the Pre-rTMS testing section of the experiment, rTMS will be applied to either the right or the left frontal area of your forehead.

To receive the rTMS, a doctor or technician will first place the stimulator on your head. You may hear a clicking sound and possibly feel a tapping sensation on your scalp. The stimulator will be adjusted to give just enough energy to send electromagnetic pulses through your scalp to make your fingers twitch. The amount of energy required to make your thumb twitch half the time is called the "motor threshold." Everyone has a different motor threshold and the treatments are given at an energy level that is based on the individual's motor threshold. Once these technical settings are decided, you will receive a treatment to the left side of your head that will last about 20 minutes. You may feel mild discomfort at the place where the coil is placed.

After completion of the rTMS, we will place the electrode cap and eye tracking headgear on again and repeat the picture testing using another set of pictures and sounds with the same characteristics as the first set. We will measure your brain waves again with the electrode cap as you make choices based on the pictures and sounds. After completing these judgments, the testing session is complete.

In addition, we will also repeat some of the performance tests that were administered to you immediately after the screening. This segment will last about 15 minutes.

We will ask you to come back two more times and perform the same set of tests and rTMS, but on these occasions we will be applying the rTMS to the other side of the forehead or applying "sham" rTMS. Sham rTMS feels and sounds the same as a "real" rTMS session, but there will be no magnetic effect on the brain because the magnetic field is blocked by the sham (fake) coil.

Participants
Initials: _____

Evaluations during the Research: At the end of each week you will visit with staff in detail about possible concerns or ill effects. At the end of the third session you will be asked to respond to a few different questionnaires. These questionnaires will look at your mood and mental functioning.

Participants
Initials: _____

Investigational Procedures: The magnetic stimulator is considered an investigational (experimental) device by the FDA for assessing a hyperarousal response to emotional stimuli.

Number of Participants: The investigators plan to enroll and complete approximately 45 participants in this research project. There will be 15 young healthy controls, 15 veterans without over-arousal to emotional stimuli, and 15 subjects with over-arousal to emotional stimuli.

Possible Risks: Though rare, the greatest possible risk from the electrode cap is scalp irritation resulting from the cap of sensors. To minimize this risk we will irritate the scalp as little as possible during the task. If you become excessively uncomfortable let the experimenter know and we can stop the experiment. Other possible risks are boredom or fatigue. If you experience either of these side effects let the experimenter know and we can take a break before resuming the activity.

You will be asked about several things which could be possible risks to your health during the use of rTMS (Repetitive Transcranial Magnetic Stimulation), and that will prevent your participation in this study. Some of the information that we ask you about may cause anxiety or discomfort. These include: A history of eating disorders, a history of a neurological disorder, including brain tumors, seizures, stroke, blood vessel abnormalities in your brain, dementia, Parkinson's disease, Huntington's chorea, or multiple sclerosis. We will also ask about anything which could increase your risk of having a seizure, including a history a head trauma with a loss of consciousness (blacked out) for more than 5 minutes, or the current use of certain medications, the presence in your body of cardiac

pacemakers, implanted medication pumps of any sort that would increase the risk of rTMS, or a history of bad heart disease, the presence of any metal objects in or near your head which cannot be safely removed for the duration of this study.

Other possible risks seen with magnetic stimulation are local pain, headache, including migraine, worsening of depression, brief dizziness, brief changes in attention and thinking, induced mania, seizure, or brief hearing loss. There may be other long term risks that are currently unknown.

Pain at rTMS site/neck pain/headaches/mild burn: During the treatment, you may feel buzzing, tapping, or painful feelings at the rTMS site, but patients who have had magnetic stimulation usually report these to be mild. You may choose to take acetaminophen (Tylenol) to decrease these sensations. There is a slight risk of scalp burn from the procedure. There have been two reports of a patient experiencing a first degree burn (similar to a sunburn). If you experience a sensation of heat at any point during rTMS, inform the doctor or technician right away. Treatment will be stopped at once if this occurs.

Temporary numbness of the face, lasting for 5 weeks after treatment with rTMS (Repetitive Transcranial Magnetic Stimulation) but then stopped, was reported in one patient.

Seizure Risk: Out of over 1,000 people given magnetic stimulation to date, ten people (less than 1%) have had seizures related with different treatment methods. Certain medications may increase the risk of seizure, and people taking these medications are excluded from participating in this research. Your rTMS will be administered in an area where trained medical personnel and equipment will be available if a seizure should occur. In the event that you have a seizure, the study staff will stop the session at once and make sure that you are safe during the seizure itself. This may mean moving you out of the device and to the floor. If the seizure lasts a while or requires further medical attention (all previously reported seizures from rTMS have been self-limited requiring no intervention), the study staff will make sure that this treatment is provided to you as quickly as possible. This may mean removing any liquid or other materials from your mouth and, if necessary, providing medications to help stop the seizure. You will be watched for a period of time after the seizure to make sure you are feeling well, and someone will be asked to drive you home that day. Having a seizure includes a possible effect on your future employability, insurability, and ability to drive. Should you experience a seizure that is related to magnetic stimulation, your doctor will provide you with a letter stating that the

seizure was produced under experimental conditions and that there is no reason to expect another occurrence.

If you are taking medication that may lower the seizure threshold like lithium, stimulants, buproprion, tricyclic antidepressants, antipsychotics, and theophylline, you will be excluded from the study. You may currently be taking other medications for medical reasons. Each medication and supplement will be checked to ensure that it does not significantly lower the seizure threshold. If the medication is determined to lower the seizure threshold, you will be excluded from the trial.

Hearing loss: Because the stimulator can emit a loud noise, and there is a risk of temporary hearing loss, you will wear protective earplugs during treatment. Studies have shown this preventative measure to be effective in protecting hearing.

Other Risks Associated with the Study: As a result of giving a urine sample, you may find out that you are pregnant. Also, treatment with the Magstim rTMS (Repetitive Transcranial Magnetic Stimulation) Stimulation System may involve other risks that are not known at the present time. The long-term effects of rTMS (Repetitive Transcranial Magnetic Stimulation) are not known. The performance capacity tests involve no more risk than you would encounter in daily life. You may become slightly fatigued or concerned with how well you seem to be executing the tasks.

Risks to an Embryo, Fetus, or Breast-Fed Infant: A woman who is pregnant or is breast-feeding an infant should not take part in this research.

It is not known whether magnetic stimulation may harm an embryo or fetus or an infant who is breast-feeding. It is not known whether magnetic stimulation may lead to birth defects.

The effect on pregnancy and the unborn fetus are unknown and pregnant women are excluded from the study. Women of child bearing potential must use a medically acceptable birth control method during the trial.

Pregnancy test: A pregnancy test will be performed for any woman is capable of bearing children who wishes to take part in this research. A study doctor will ask for the date when a woman's last monthly period started.

Avoiding pregnancy: Whether you are a woman or a man, you should ask study personnel about the effective means to prevent a pregnancy during participation. Ask your study personnel how long you must avoid becoming pregnant or fathering a child after you complete all study procedures.

If you change your method of avoiding pregnancy or fathering a child during the research, you must notify your study personnel as soon as possible.

The company that makes the stimulator and the study personnel strongly recommends that participants in this research use medically acceptable birth control method during this study.

Pregnancy during participation in this research: If you are a woman and you suspect pregnancy during this research, you must tell your study personnel immediately. Your participation in the research will stop. Your study personnel will report information about your pregnancy, delivery, and the baby's first two months of life to the device manufacturer of Magstim.

Unforeseen risks: Treatment with the Magstim rTMS (Repetitive Transcranial Magnetic Stimulation) Stimulation System may involve other risks that are not known at the present time. The long-term effects of rTMS (Repetitive Transcranial Magnetic Stimulation) are not known.

How you can help reduce some of the risks: During your participation in this research, your study personnel will watch closely to determine whether there are problems that need medical care. It is your responsibility to do the following:

- Ask questions about anything you do not understand.
- Keep appointments.
- Follow the study doctor's instructions.
- Let your study doctor know if your telephone number changes.
- Tell your study doctor before you take any new medication even if it is prescribed by another doctor for a different medical problem.
- Tell your regular doctor about your participation in this research.
- Talk to a family member or friend about your participation in this research.
- Carry information about the research in your purse or wallet.

What to do if you have problems: If you have problems, such as unusual symptoms or pain, at any time during your participation in the research, your

study doctor can suggest treatment. Please report the problem to your study doctor promptly. Telephone numbers where he/she may be reached are listed on the first page of this consent form.

If you suddenly have a serious problem (such as difficulty breathing) or severe pain, go to the nearest hospital emergency room, or call 911 (or the appropriate emergency telephone number in your area). Tell emergency personnel about your participation in this research. Ask them to telephone your study doctor immediately.

Possible Benefits to the Participant: Participation in this study may have no direct benefit to you. If you experienced overarousability to emotional stimuli, participating in this study may or may not improve this condition. It is most likely that there will be no change in your condition. Your condition will be closely monitored throughout all parts of the experiment.

Benefit to other people: In the future, other people with over-arousal to emotional stimuli could benefit from the results of this research. Information gained from this research could lead to improved medical care for persons experiencing hyperaroused responses to emotional stimuli. However, your study personnel will not know whether there are benefits to other people until all of the information obtained from this research has been collected and analyzed. Your participation in this study may help researchers to understand the process of being over-vigilant and over-responding to emotional stimuli, and this may eventually help other patients in the future.

Alternatives to Participation: You do not have to take part in this research. Please ask your study doctor as many questions as you wish. The doctor's answers to your questions could help you decide whether to participate in this research. If you decide to take part in research now, and later change your mind, you may stop your participation in the research then.

The Study Investigator's Decision to Stop Your Participation: Your study investigator may stop your participation in this research without your permission under any one of the following conditions:

- You have a serious adverse event (i.e., seizure).
- Your medical problem becomes worse.
- Your study doctor believes that participation in the research is no longer safe for you.
- Your study doctor believes that other treatment may be more helpful.

- The device manufacturer or the FDA stops the research for the safety of the participants.
- The funding agency (Department of Defense) or device manufacturer cancels the research.
- You are unable to keep appointments or to follow your study investigator's instructions.

Procedures after Stopping Participation in this Research: If you or the study investigator stops your participation in the research, it is your responsibility to do the following:

- Let your study investigator know immediately that you wish to withdraw from the research.
- Return to the research center for tests that may be needed for your safety.
- Discuss your future medical care with your study investigator and/or your regular doctor.

Reimbursement of Expenses [or Payments to Participate]: You will be compensated for your participation in this study. For participation, you will receive $25 for completing the initial pre-rTMS screening. After each session where you receive rTMS, you will receive $50. If you complete the entire study, an additional $25 will be awarded, resulting in a total payment of $200 for participating in the entire study.

There are no additional costs to you or your insurance company for participating in this study.

COSTS TO YOU: The study funding agency will cover expenses for the procedures that are part of this research.

Neither you, nor your insurance provider, will be charged for anything done only for this research study (i.e., the Screening Procedures, Experimental Procedures, or Monitoring/Follow-up Procedures described above).

There are no funds available to pay for parking expenses, transportation to and from the research center, hospitalization for depressive symptoms, suicide attempts, manic episodes, medications, lost time away from work and other activities, lost wages, or childcare expenses.

Voluntary Participation: You have the right to agree or refuse to participate in this research. If you decide to participate and later change your mind, you are free to discontinue participation in the research at any time. Such withdrawal will

not affect your current or future treatment, or any benefits to which you are otherwise entitled. You will be asked to complete the interview and other procedures of the final visit.

Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. Refusal to participate will not affect your legal rights or the quality of health care that you receive at this center.

New Information: Any new information which becomes available during your participation in the research and may affect your health, safety, or willingness to continue in the research will be given to you.

Records of Participation in this Research:
Information Stored at the University of Texas at Dallas
All of the information participants provide to investigators as part of this research will be protected and held in confidence within the limits of the law and institutional regulation.

Information about you that is collected for this research study will remain confidential unless you give your permission to share it with others, or if we are required by law to release it.

Personal identifying information will be kept separate from your data in a locked file cabinet. Only the investigators directly involved with this research project who have been trained in methods to protect confidentiality will have access to these files. Your actual data will be coded so that it cannot be linked to you without the locked file information.

Information Available to Others:
Members and associated staff of the Institutional Review Board (IRB) of the University of Texas at Dallas may review the records of your participation in this research. An IRB is a group of people who are responsible for assuring the community that the rights of participants in research are respected. A representative of the UTD IRB may contact you to gather information about your participation in this research. If you wish, you may refuse to answer questions the representative of the IRB may ask.

You should know that certain organizations that may look at and/or copy your medical records for research, quality assurance, and data analysis include:

- Department of Defense

- Representatives of government agencies, like the U.S. Food and Drug Administration (FDA), involved in keeping research safe for people; and

In addition to this consent form, you will be asked to sign an "Authorization for Use and Disclosure of Protected Health Information." This authorization will give more details about how your information will be used for this research study, and who may see and/or get copies of your information.

Publications Associated with this Research: The results of this research may appear in publications but individual participants will not be identified.

Compensation for Injury: Compensation for an injury resulting from participation in this research is not available from the University of Texas at Dallas. However, you retain your legal rights during participation in this research.

Contact People: Participants who want more information about this research may contact any of the investigators listed at the top of page 1 of this document. Participants who want more information about their rights as a participant or who want to report a research related injury may contact:

Sanaz Okhovat, Assistant Director of Research Administration and Compliance

Phone 972-883-4579
Fax 972-883-2310
Email: sanaz.okhovat@utdallas.edu

Additional information, including the nature and details of the researcher's or the research entity's financial interest, are available upon request.

Signatures

A participant's signature indicates that they have read, or listened to, the information provided above and that they have received answers to their questions. The signature also indicates that they have freely decided to participate in this research and that they know they have not given up any of their legal rights.

_____
Participant's Name (printed)

_____ _____
Participant's Signature Date

_____
Name of Researcher Obtaining Consent

_____ _____
Signature of Researcher Obtaining Consent Date

_____
Name of Witness

_____ _____
Signature of Witness Date

The University of Texas at Dallas
Institutional Review Board

JUN 20 2007 JUN 19 2008

Approved Expires

THE UNIVERSITY OF TEXAS AT DALLAS

IRB Application for Expedited or Full Board Review
for
Research Procedures Involving Human Participant

CONTACT INFORMATION

Project Title: Repetitive Transcranial Magnetic Stimulation (rTMS) to Temporarily Reduce Over-Arousal to Emotional Stimuli

Principal Investigator	
Name John Hart, Jr	Degree(s) MD
University Title Professor	Department Behavioral and Brain Sciences
Campus Phone No. 972-883-3212	E-Mail Address jhart@utdallas.edu
Campus Mailing Address Center for BrainHealth 2200 W. Mockingbird Lane Dallas, Tx 75235	Campus Mail Code BH

CO-Investigator or Faculty Sponsor	
Name Sandi Chapman	Degree(s) PhD
University Title Professor	Departments Behavioral and Brain Sciences
Campus Phone No. 972-883-3404	E-Mail Address schapman@utdallas.edu
Campus Mailing Address Center for BrainHealth 2200 W. Mockingbird Lane Dallas, Tx 75235	Campus Mail Code

Primary Contact Person		
Name Patricia Sinclair Moore	Campus Phone No. 214-905-3007	E-Mail Address psm072000@utdallas.edu

FEDERAL DEFINITION OF RESEARCH

Department of Health and Human Services and other Federal Regulations require that the IRB must determine whether the proposed study or data collection meets the federal definition of research.

Federal regulations state that the protocol of all research projects must be submitted for IRB review and can only be conducted after IRB approval. Research is defined as, "a systematic investigation, including research development, testing and evaluation, designed to develop or contribute to general knowledge".

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If you do not believe that your project is research as defined above please complete the form to determine research status located at http://www.utdallas.edu/research/rc_irb_forms.htm

FUNDING

Check all of the appropriate boxes for funding sources for your research, including sources of pending support:

☒ Extramural
☐ Callier Excellence
☐ Department
☐ Gift: (Please describe)
☐ Other: (Please describe)

P.I. of Contract or Grant:
Funding Source: Department of Defense
Contract or Grant No.: Log Number 06177002

Contract or Grant Title: Interdisciplinary Studies on the Combat Readiness and Health Issues Faced by Military Personnel.

Complete copies of each funding application must be forwarded to the IRB at the time the funding application is submitted or with the original IRB application.

If using an IDENTICAL protocol for more than one extramural funding proposal, list all of the funding sources below. Attach an additional sheet if more space is needed.

P.I. of Contract or Grant:
Funding Source:
Contract or Grant No.:
Contract or Grant Title:

P.I. of Contract or Grant:
Funding Source:
Contract or Grant No.:
Contract or Grant Title:

Investigators are responsible for informing the IRB immediately of any change in grant application or funding status that may occur during the course of the investigation.

Will you also submit application(s) to other IRBs for approval of the same project?
☒ Yes ☐ No

If yes, provide the following for each IRB that is expected to review any part of this project:

Institution	IRB Contact Person	Phone Number
University of Texas Arlington	Jan Parker	817-235-3542
Street Address		
City	State	Zip

The University of Texas at Dallas
Institutional Review Board

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JUN 19 2008 Expires

PROTOCOL SUMMARY

Instructions:

Use non-professional language and address each part separately to describe your protocol. Attaching sections of a grant application or proposal is not an acceptable substitute. Provide sufficient information for effective review by all members of the IRB, including non-specialists. Define all abbreviations and terms that are not part of common language.

Narrative

What is the goal of the investigation?
The goal of this study is to provide information regarding the use of repetitive Transcranial Magnetic Stimulation to reduce a hyperarousal response in individuals where their overactive response to stimuli in their environment impairs their ability to function.

Describe previous studies that form the basis for the proposed research.
What information do you expect to obtain and how will the obtained knowledge be applied?
Patients with head injury, anxiety disorders, phobias, and post-traumatic stress disorder have overreactions to elements in their environment that can produce anxiety, fear, and impaired attention/concentration. These hyperarousal responses have been resistant to medical treatment or the medications produce significant sedating effects. Neither of these side effects impair the activities of daily living of these patients. Our preliminary studies have shown that treatment with repetitive transcranial magnetic stimulation, a noninvasive intervention with minimal secondary risks, can be applied to temporarily alleviate these symptoms. These studies have suggested that use of repetitive transcranial magnetic stimulation may be developed to alleviate or prevent development of these hyperarousal symptoms without having to use long term medical management. The proposed studies are to be used to further develop this approach.

Research Locations

Where will the research be conducted?

- ☐ UTD – Richardson campus
- ☐ UTD – Callier campus
- ☒ Other

Institution	IRB Contact Person	Phone Number
Center for BrainHealth		
Street Address		
City	State	Zip

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METHODS AND PROCEDURES FOR THE COLLECTION OF NEW DATA

Describe each activity in which participants will be involved. State the expected duration of each activity, intervals between activities (if any), and the overall time commitment by the subjects.

Mark one or more of the following that apply.

- ☐ Interviews
- ☐ Survey/questionnaire
- ☒ Behavioral observation
- ☐ Waiver of consent
- ☐ Deception
- ☒ Clinical studies

- ☐ Potential development of commercial product
- ☒ PI or Co-PI is Health Service Provider
- ☒ Health Service Provider is certified/licensed by the State of Texas

- ☒ Biomedical Procedure

- ☐ Biological specimens
- ☐ Venipuncture (<550cc)
- ☒ Investigational devices
- ☐ Microorganisms
- ☐ Use of Controlled Substances or other potentially addictive medication

- ☐ Genetic research
- ☐ Study of HIV/AIDS positive individuals

For research involving contact with participants, describe the place and setting where this type of procedure will take place:

The location of the procedure will be in the Center for BrainHealth.

How is the contact to be conducted?

- ☒ In Person, One on One
- ☐ Small Group (2-5)
- ☐ Moderate Size Group (6-15)
- ☐ Large Group (>15)
- ☐ Paper and Pencil
- ☐ Other
- ☐ Telephone
- ☐ Computer in Laboratory
- ☐ E-mail
- ☐ US Mail

Submit copies of all survey instruments, questionnaires, interview scripts/outlines, summary forms, etc. to the IRB to be reviewed and approved at the time of the IRB review. Any copyrighted materials must be accompanied by the authorization to copy by the copyright holder.

The University of Texas at Dallas
Institutional Review Board

Name all documents that will accompany this application.

Questionnaire(s):
Interview Script(s):
Published Test Protocols:
Record/Chart Review Information:
Other: 1) The study protocol will be submitted to describe the reasoning, pilot work, and procedures.

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2) An appendix describing the human motor performance apparatus to be used.
3) A memo that this protocol will also be submitted to the UT Arlington IRB.

Selection procedures for participants.

☒Participants will be fully informed about the selection criteria and the selection procedure.
☐Participants will not be fully informed about the selection procedure.
Explain:

Will a Control Group be used?
☒ Yes ☐No

If yes:
☒ Participants will be informed that they may be a member of a control group.
☐ The individual participants who will not be a member of a control group will not be informed about that.
Explain:

Please describe in detail the standard professional and/or clinical procedures to be used.
1. Human Motor Performance Capacity Tests are a standardized set of motor performance measures to assess human motor speed and directionality of motor movement of the hands
2. Color photographs that will be presented for a category identification task.
3. Electroencephalography (EEG) which will be used to record the subject's brain waves to assess
4. An eyetracker is a noninvasive system that records the movement of the eyes by the use of light reflection as a subject views a stimulus.

Describe the experimental procedures that will be performed exclusively for research purposes.
Repetitive Transcranial Magnetic Stimulation unit: TMS uses a handheld electrical coil that is positioned on the scalp. The coil is powered by capacitors that send a pulse of current through the coil. The pulse of electrical current creates a magnetic pulse that passes unimpeded through the skull to the brain. The magnetic field pulse induces electric fields in the brain that depolarize the neurons. The ability to non-invasively stimulate the brain has led to numerous areas of investigation. When the pulses are delivered repeatedly, it is referred to as repetitive transcranial magnetic stimulation (rTMS) (George and Belmaker 2000). The proximity of the brain to the time-varying magnetic field will result in current flow in neural tissue, thereby activating or deactivating underlying cortex (George et al., 1999). It is estimated that there is an approximately 2-cm magnetic field penetration from the scalp surface. rTMS thus offers a noninvasive method for altering excitability of the brain.

METHODS AND PROCEDURES FOR THE USE OF ARCHIVAL DATA

Will archival data be used?

☐ Yes ☒No

If no, go to **Data Collection, Storage and Confidentiality**

If yes, please continue.

Attach a copy of a data coding form showing how you will record the relevant information.

What is the source of the data or biological specimens to be used?

How was the information originally collected?

What was the population from which these records were derived? (Age, gender, etc.)

Do the individuals (or their legally authorized representative) from which data are to be used know that these records exist?

☐ Yes ☐No ☐Not Sure

If no, explain:

What specific information will you extract?

If this record or specimen became publicly available, could it have negative psychological, physical, economic, sociological or legal consequences for the participant from which it originated?

☐ Yes ☐No

If yes, describe the potential negative consequences.

Data Collection, Storage and Confidentiality

Will identifiers or links to an identifier of the participants be stored?

☒Yes ☐No

If yes, what information that could be linked to the participants will be recorded?
Name, age, gender, race, education, handedness, medical diagnoses, current medications.

Will you obtain any information containing personally identifying information?

☒Yes ☐No

If no, please move on to the **PARTICIPANTS SECTION**.

If yes, please continue with the following questions:

If information with personal identifiers will be accessed, will the participants provide consent for storing of personal data or biological specimens in connection with the research?

☒ Yes ☐ No

If yes, attach a copy of an informed consent.

If no, answer the following questions in order to provide justification for a waiver of informed consent.

Why does the proposed use of existing data or biological specimens present no more than minimal risk to the participants?

Why could the research not practicably be carried out with an informed consent from the participants?

Why will a waiver of informed consent not adversely affect the rights and welfare of the participants?

Will the participants be informed about which information is recorded and stored?

☐ Yes ☐ No

If no, explain:

PARTICIPANTS

Number of Participants

How many subjects from UTD will be included?
30 subjects total with the possibility that some of the 10 normal controls will be from UTS

Will subjects be recruited from other locations?
☒ Yes ☐ No

If yes, state how many participants are anticipated at each site.
10 veterans from the VA or community and 10 depressed, head injured or anxious adults from investigators' clinics.

Selection Criteria

Equitable inclusion of both men and women of all ages, and individuals from diverse racial/ethnic backgrounds, is important to assure that they receive an equal share of the benefits of research and that they do not bear a disproportionate share of its burdens. Participation of adult participants of both genders and diverse racial/ethnic backgrounds should not be restricted without medical or scientific justification.

How will you determine whether a volunteer can be included or not?
Inclusion criteria for subjects

- Male and female outpatients aged 29-35 years old
- Cognitively intact (Folstein MMSE score >24).
- Clinically competent to give informed written consent

Exclusion Criteria for subjects

- History of epilepsy or seizure disorder, mass brain lesions, cerebrovascular accident, metal in the skull, a history of major head trauma, or any neurologic condition likely to increase risk of rTMS.
- Diagnosis of Post-Traumatic Stress Disorder or phobia
- Lifetime history of schizophrenia, schizoaffective, or other psychotic disorder, bipolar disorder type I or II, dementia, dissociative disorders, and sexual and gender identity disorder
- Personality disorder that makes participation in the trial difficult
- History of Substance Abuse or Dependence (DSM-IV) in the last year except nicotine and caffeine
- Taking any medication that significantly lowers the seizure threshold (e.g. lithium, stimulants, buproprion, TCAs, antipsychotics, theophylline, etc.)
- Unstable medical conditions that precludes safe participation in rTMS treatment trial
- Known or suspected pregnancy
- Women of child-bearing potential not using medically accepted form of contraception when engaged in sexual intercourse
- Any metal or device implants that would increase risk of rTMS
- Unable to determine the motor threshold in the subject
- History of Vagus Nerve Stimulation

Is any aspect of the selection of the subjects based on any of the following?

☒ Yes ☐ No

If yes, select:

☒ Age
☐ Gender
☐ Racial/ethnic origin
☒ Physical condition (Please List) See above.
☒ pregnancy or childbearing potential
☐ mental condition

Describe the selection criteria and their justification (for instance, if only women are included, explain the rational for excluding men).

The age group is relevant for those with hyperarousal disorders. The physical condition will be normal controls or a physical condition related to having a hyperarousal disorder. rTMS safety has not been fully investigated in those who are pregnant and thus pregnant woman are being excluded as an extra precaution.

What are the qualifications and training of the staff who will determine inclusion and exclusion?

Dr. John Hart, a behavioral neurologist, and Dr. Andy Kozell a psychiatrist with extensive experience in the use of rTMS will review the neurological and psychiatric criteria.

Characteristics of Participants

Participants can be vulnerable for multiple reasons. Some examples of vulnerable participant include: children, the elderly, pregnant women, fetuses, cognitively impaired individuals, emotionally impaired persons, terminally ill patients, institutional residents, prisoners, parolees, non-English-speaking participants, and UTD students/staff.

To which of the following categories do the participants in this research belong?

- ☒ Adults
- ☐ Babies and Toddlers (0-3)
- ☐ Young Children (4-10)
- ☐ Youth (11-12)
- ☐ Adolescents (13-18)
- ☐ Elderly (>65)
- ☐ Families (Parents w Child)
- ☐ Prisoners or parolees
- ☒ UTD Students/Staff
- ☐ Children in Daycare
- ☐ Children in School
- ☐ Teachers or Staff in Schools
- ☒ Clinic or Hospital Patients
- ☐ Clinic or Hospital Staff
- ☐ Institutional residents

- ☐ Person with language/hearing disability
- ☒ Person with emotional disability
- ☐ Person with cognitive disability
- ☐ Person with physical disability
- ☐ Comatose
- ☐ Cancer patients
- ☐ Terminally ill
- ☐ Non English speaker

(If you have checked one of the above boxes and are in need of assistance in accommodating a person with disabilities during the research activities please contact the IRB office.)

- ☐ Pregnant women
- ☐ Fetuses
- ☐ Other
- ☐ Women undergoing in vitro fertilization

Will any vulnerable participants be included?

☐ Yes ☒ No

If yes, what is the justification for the inclusion of each vulnerable group named.

PARTICIPANT IDENTIFICATION AND RECRUITMENT

Method of Recruitment of Subjects

The identification and recruitment of participants must be ethically and legally acceptable and free of coercion. Procedures used to recruit participants should be designed to reach diverse populations. For example, vulnerable participants, such as persons in nursing homes or institutions, should not be recruited merely for the sake of convenience.

- ☒ Advertisement
- ☐ Verbal scripts for face-to-face meeting
- ☐ Letters to potential participants
- ☐ Detailed Plan and Form for Records Review
- ☐ Other Please List:
- ☐ Telephone Script
- ☐ E-Mail
- ☐ Web-Based

Provide a detailed description of each recruitment method that is used.

Recruitment

Participants will be recruited by other studies that are being conducted in emotional memory processing using ERP. There are normal studies of young adults, veterans of the Gulf War, and individuals with depression. In those studies, there are parallel sets of stimuli being used to assess emotional processing with ERP. Those individuals that are identified with a hyperarousal response and those with a normal and hypoarousal response will be recruited for this study. Advertising campaigns that include newspapers, billboards, brochures, flyers, and contact with colleagues have been successful in recruiting for these types of studies.

A research coordinator performs an initial phone screening, and if determined eligible, the potential subject is scheduled for a face-to-face evaluation with the physicians in the study.

METHODS TO PROTECT CONFIDENTIALITY

The principal investigator/faculty sponsor is responsible for taking all necessary steps to maintain confidentiality of data. This includes coding data and choosing appropriate and secure ways to store data to prevent unauthorized access to the data.

How will you protect privacy and confidentiality?

- ☐ No identifying information will be collected
- ☒ Data will be securely stored.

UTD Bldg: CBH Rm: Secure files room on third floor

- ☒ Identifying information will be removed
- ☒ Data will be securely stored in a location separate from consent documents.
- ☒ Access to data will be available to less than 5 individuals.
- ☒ Password/Security code will restrict access to data.
- ☒ Random ID codes will be assigned.
- ☐ Key to Match Random Codes will not exist.
- ☒ Key to Match Random Codes will be stored separate from data.
- ☒ Key to Match Random Codes will only be accessed by investigators for analysis.
- ☒ All research staff will be trained in research ethics and methods to protect confidentiality.
- ☐ Encryption of all recorded data
- ☐ Other

Will data with any participant identifiers be released to anyone?

☐ Yes ☒ No

If no, please move on to the **RISK/BENEFIT ASSESSMENT SECTION**.
If yes, please continue with the following questions:

Specify the person(s) or agency to whom this information will be released:
Specify when this release will take place:

What will happen to the data after the data analysis is complete?

- ☐ Data will be destroyed _____ years after completion of the study
- ☐ Data will be stored an unspecified length of time

RISK/BENEFIT ASSESSMENT

A reasonable person would consider it to be important to know the risk of harm or discomfort when deciding whether to participate in the research project.

Potential Risks of Discomfort and Harm

Are there risks of physical harm or discomfort associated with the research?

☒ Yes ☐ No

If yes, describe:

You will be asked about several things which could be possible risks to your health during the use of rTMS (Repetitive Transcranial Magnetic Stimulation), and that will prevent your participation in this study. Some of the information that we ask you about may cause anxiety or discomfort. These include: A history of eating disorders, a history of a neurological disorder, including brain tumors, seizures, stroke, blood vessel abnormalities in your brain, dementia, Parkinson's disease, Huntington's chorea, or multiple sclerosis. We will also ask about anything which could increase your risk of having a seizure, including a history a head trauma with a loss of consciousness (blacked out) for more than 5 minutes, or the current use of certain medications, the presence in your body of cardiac pacemakers, implanted medication pumps of any sort that would increase the risk of rTMS, or a history of bad heart disease, the presence of any metal objects in or near your head which cannot be safely removed for the duration of this study.

Other possible risks seen with magnetic stimulation are local pain, headache, including migraine, worsening of depression, brief dizziness, brief changes in attention and thinking, induced mania, seizure, or brief hearing loss. There may be other long term risks that are currently unknown.

Pain at rTMS site/neck pain/headaches/mild burn: During the treatment, you may feel buzzing, tapping, or painful feelings at the rTMS site, but patients who have had magnetic stimulation usually report these to be mild. You may choose to take acetaminophen (Tylenol) to decrease these sensations. There is a slight risk of scalp burn from the procedure. There have been two reports of a patient experiencing a first degree burn (similar to a sunburn). If you experience a sensation of heat at any point during rTMS, inform the doctor or technician right away. Treatment will be stopped at once if this occurs.

Temporary numbness of the face, lasting for 5 weeks after treatment with rTMS (Repetitive Transcranial Magnetic Stimulation) but then stopped, was reported in one patient.

Seizure Risk: Out of over 1,000 people given magnetic stimulation to date, ten people (less than 1%) have had seizures related with different treatment methods. Certain medications may increase the risk of seizure, and people taking these medications are excluded from participating in this research. Your rTMS will be administered in an area where trained medical personnel and equipment will be available if a seizure should occur. In the event that you have a seizure, the study staff will stop the session at once and make sure that you are safe during the seizure itself. This may mean moving you out of the device and to the floor. If the seizure lasts a while or requires further medical attention (all previously reported seizures from rTMS have been self-limited requiring no intervention), the study staff will make sure that this treatment is provided to you as quickly as possible. This may mean removing any liquid or other materials from your mouth and, if necessary, providing medications to help stop the seizure. You will be watched for a period of time after the seizure to make sure you are feeling well, and someone will be asked to drive you home that day. Having a seizure includes a possible effect on your future employability, insurability, and ability to drive. Should you experience a seizure that is related to magnetic stimulation, your doctor will provide you with a letter stating that the seizure was produced under experimental conditions and that there is no reason to expect another occurrence.

If you are taking medication that may lower the seizure threshold like lithium, stimulants, buproprion, tricyclic antidepressants, antipsychotics, and theophylline, you will be excluded from the study. You may currently be taking other medications for medical reasons. Each medication and supplement will be checked to ensure that it does not significantly lower the seizure threshold. If the medication is determined to lower the seizure threshold, you will be excluded from the trial.

Hearing loss: Because the stimulator can emit a loud noise, and there is a risk of temporary hearing loss, you will wear protective earplugs during treatment. Studies have shown this preventative measure to be effective in protecting hearing.

Other Risks Associated with the Study: As a result of giving a urine sample, you may find out that you are pregnant. Also, treatment with the Magstim rTMS (Repetitive Transcranial Magnetic Stimulation) Stimulation System may involve other risks that are not known at the present time. The long-term effects of rTMS (Repetitive Transcranial Magnetic Stimulation) are not known. The performance capacity tests involve no more risk than you would encounter in daily life. You may become slightly fatigued or concerned with how well you seem to be executing the tasks.

Are there risks of psychological harm or discomfort associated with the research procedure?

☐ Yes ☒ No

If yes, describe:

Are there risks of social harm to the participants associated with the research?

☐ Yes ☒ No

If yes, describe:

Are there economic risks associated with the research?

☐ Yes ☒ No

If yes, describe:

What is your assessment of the overall risk classification of this research?

☐ Minimal ☒ More than Minimal ☐ Unknown

Minimizing Risks

All risks and discomfort must be minimized to the greatest extent possible. According to DHHS/FDA Regulations minimal risk means "The probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests." When the risk associated with a new procedure or product are unknown, they cannot be classified as "minimal."

If the risk is estimated to be greater than minimal, a plan for Emergency Care an/or Compensation must be made for research-related injury and information about such procedures must be included in the Consent Forms that are signed by the participants.

How will you minimize risks or discomfort?

- ☒ Monitor the experiments by professional staff.
- ☒ Provide opportunities for rest or breaks.
- ☒ Withdrawal of participant based on specific criteria
- ☒ Remind participant of his/her opportunity to stop or withdraw.
- ☐ Modification of process
- ☐ Other

Potential Benefits

The Federal Office for Human Research Protection (OHRP) defines direct benefit as a benefit other than that derived from making a contribution to science.

What are the potential benefits to participants from their participation in this research?

- ☒ No direct benefit.
- ☐ A direct benefit to the participant(s).

Explain:

Societal benefits generally refer to the advancement of science or knowledge and/or possible future benefit to individuals with similar concerns or disorders.

Describe the potential benefits to society from this research?

In the future, other people with over-arousal to emotional stimuli could benefit from the results of this research. Information gained from this research could lead to improved medical care for persons experiencing hyperaroused responses to emotional stimuli. However, your study personnel will not know whether there are benefits to other people until all of the information obtained from this research has been collected and analyzed. Your participation in this study may help researchers to understand the process of being over-vigilant and over-responding to emotional stimuli, and this may eventually help other patients in the future.

Risk/Benefit Ratio

The potential benefits of research must justify the risks to human participants. Some risks may not be reasonable, no matter how important the potential benefits. The risk/benefit ratio of the research must be at least as favorable for the participants as that presented by standard treatments for their condition. When comparing the risk/benefit ratio of research with that of available alternatives, the alternative of doing nothing, or "watchful waiting," should be included in the analysis.

What is your assessment of the risk/benefit ratio of the proposed research, compared with that of available alternatives?

Presently, there are no available alternatives. In the future, the animal models and our preliminary studies suggest that use of rTMS after a traumatic event (car accident, rape, physical abuse, sexual abuse, war trauma) can prevent the development of hyperarousable responses. If not, repeated use of 1 Hz rTMS, which has little risk, could lead to a subject developing deconditioning to arousing stimuli. If this were the case, then patients would be prevented from developing these debilitating conditions or have a non-medication treatment. These benefits outweigh this minimal or mildly more than minimal risk.

FINANCIAL CONSIDERATIONS

The FDA encourages a prorated system of payment whereby participants who do not finish the protocol are paid in proportion to the part completed. The amount of payment must be justified and not constitute undue inducement of the participant to participate in the research. If a non-prorated system of payment will be used, this should be justified in this section.

Payment for Participation:

☐ Participants will not be reimbursed for expenses or receive any other payment.
☐ Participants will be paid a fee of $ _____ for reimbursement of travel or other expenses.
☐ Participants will be paid a fee of $ _____ for initial enrollment.
☒ Participants will be paid a fee of $ 25 for screening and $50 for each rTMS session which will total $175 for the study.
☒ Participants will be paid an additional fee of $ 25 for completing the research protocol.
☐ Participants will receive a valuable service for their participation:
Describe the service and its value:
☐ Other rewards or inducements will be used to encourage participation:

☐ Participants will receive a potential reward:
Describe this potential reward, and the likelihood a participant will receive it:

Financial Obligations of the Participants

This section should clarify if the participant will incur any costs as a result of participating in this research project. If clinical service is associated with the research, specify who will have financial responsibility for routine clinical care. State clearly whether participants' costs may be increased as a result of additional follow-up examinations and/or tests required by the research.

What financial obligations will participants incur as a result of participating in the study?
None.

Will participants have to pay for any of the treatment(s) they receive or tests performed in connection with the research?
No.

Emergency Care and Compensation for Research-Related Injury

This category of information should only be completed when the research presents **GREATER THAN MINIMAL RISK**.

If the research presents greater than minimal risk, what emergency care is available in case of research-related injury? During the time of the rTMS, there will be a neurologist or psychiatrist experienced with the procedure on site. There will be oxygen available if the patient were to have a seizure and the physicians involved are experienced in dealing with patients with a seizure. Tylenol will be available on site to those subjects who can take this medication, if they have mild discomfort. If there are any changes in mood, the physicians on site are experienced in dealing with these types of patients.

Who will be responsible for the cost of such care? The subject.

Will participants be compensated for out-of-pocket expenses or lost wages if they suffer a research-related injury? No

INFORMED CONSENT

In research involving more than minimal risk, when capacity to consent is unclear, the capacity to consent must be determined by a physician, clinical psychologist, or by other qualified professionals. Individuals who lack the capacity to consent may participate in research only if consent is given on their behalf by a legally authorized representative.

Capacity to Consent

Will all adult participants have the capacity to give informed consent?

☒ Yes ☐ No

If not, explain the reason, describe by whom, the judgment regarding their capacity to consent will be made.

Will all child participants have parents or guardians give informed consent for their participation?

☐ Yes ☐ No

If not, explain why this is not needed or possible.

Will all child participants have the opportunity to assent to participation?

☐ Yes ☐ No

Describe how assent will be obtained in a manner that is sensitive to the developmental stage of the participants:

Who will administer the informed consent to the participants? Identify by name and training the individual(s) authorized to describe the research and obtain consent form from participants or their legal representatives.

Only those individuals authorized to solicit consent may sign the consent form confirming that the prospective participant was provided the necessary information and that any questions asked were answered to the participant's satisfaction.

Process of Consent

Consider: a) the environment and location where informed consent will be solicited; b) the timing of the process for instance the stress that may be associated with the situation); c) the involvement of someone other than the investigators to help explain the research; and d) opportunity for the prospective participants or their legal representatives to discuss participation in the research with family, friends, or their advisors before signing the consent form.

Where will the consent process take place? CBH, UTSW, VA.

What steps will be taken to avoid coercion or undue influence?
We will involve other staff to help explain the research and provide ample opportunity for the prospective participants or their legal representatives to discuss participation in the research with family, friends, or their advisors before signing the consent form.

How--and by whom--will it be determined whether the participants or their legally authorized representatives understand the information provided?

The subjects will be explained that the risks, will extremely minimal of a seizure, need to be considered. It will be explained to the subjects in detail what a seizure actually is and the implications of having one.

The risks and other exclusions will be explained if the subjects do not understand these. These will be checked off by the person obtaining consent to ensure that each question has been asked.

The subjects will be questioned about the risks of the research and asked to explain them back to the investigators to ensure that they know these risks.

Will any information about the research purpose and design be withheld from the participants?

☐Yes ☒No

If yes, explain and justify the non-disclosure and describe plans for post-study debriefing.

Any non-disclosure must be approved by the IRB. Information that a reasonable person would want to know in deciding whether to participate in the research may not be excluded and the consent procedure must be approvable under 45 CFR 46.116(d) which states: (1) the research involves no more than minimal risk to the participants; (2) the waiver or alteration will not adversely affect the rights and welfare of the participants; (3) the research could not practicably be carried out without the waiver or alternation; and (4) whenever appropriate, the participants will be provided with additional pertinent information after participation.

Consent/Assent Forms:

Specify which form(s) will be used:

☒ Adult Consent Form
☐ Parental Consent Form
☐ Child Assent Form (7-12)
☐ Youth Assent Form (usually age 13-18)

The University of Texas at Dallas
Institutional Review Board

JUN 20 2007 JUN 19 2008

Approved Expires

IRB REVIEW CATEGORY

THIS SECTION MUST BE COMPLETED

The DHHS and other Federal Regulations require that the IRB is responsible for determining whether the proposed data collection meets the federal definition of research.

Federal regulations state that all activities that are classified as research must be submitted for IRB review and approval.

Expedited category of review is reserved for research that involves minimal risk and satisfies one or more of following seven categories defined by Federal Regulation (Department of Health and Human Services). All other research involving humans must be reviewed by the FULL BOARD of the IRB.

Please mark the one that best describes your research.

- ☐ Category 1-Study of drugs or devices that do not require an IND application, are used consist w/label
- ☐ Category 2-Blood samples by finger/stick or venipuncture from healthy and nonpregnant adults <550 ml
- ☐ Category 3-Prospective bio specimens for research by non-invasive means (ex. hair or nail clippings)
- ☒ Category 4-Non-invasive procedure used in routine clinical practice
- ☐ Category 5-Materials collected previously (archival data)
  - ☐ a) for non-research purposes
  - ☐ b) for another research protocol
- ☐ Category 6-Collection of data from voice, video, digital or other recordings for research purposes.
- ☐ Category 7- Research on individuals or groups using surveys, interviews, or program evaluation, etc.

If your research project does not satisfy any of the two categories, or if the level of risk is unknown or if it involves greater than minimal risk it must be subjected to **FULL BOARD REVIEW.**

If you have additional comments for IRB Reviewers please attach them on a separate sheet.

ASSURANCES

My signature below certifies that:

I agree to comply fully with the ethical principles and regulation regarding the protection of human subjects in research.

I agree that the information provided in this form and all other supporting documents and forms are accurate and complete.

Copies of all required documentation of Consent and any data related to this research are securely stored at (UTD building and Office Number).

Principal Investigator's Signature — Date: 6/7/07

Sandra Chapman

Faculty Sponsor's Signature — Date: 6/8/07

The University of Texas at Dallas
Institutional Review Board

JUN 20 2007 — Approved

JUN 19 2008 — Expires